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STIMULATION OF IN VITRO TRANSCRIPTION BY SPERMIDINE AND POTASSIUM-CHLORIDE: A COMPARATIVE STUDY

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STIMULATION OF *in vitro* TRANSCRIPTION BY SPERMIDINE AND KCl: A COMPARATIVE STUDY

by

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To my loving wife whose perseverance and support have made the past eight years of my education possible.

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ABSTRACT

STIMULATION OF *in vitro* TRANSCRIPTION
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Stimulation of *Escherichia coli* DNA-dependent RNA polymerase activity by monovalent, divalent, and polyvalent cations and the comparative influence of KCl and spermidine on certain aspects of the termination event has been investigated.

The cations referred to in this study can be conveniently divided into classes with respect to their influence on *in vitro* transcription. Class 1 is composed of salts of monovalent cations as represented by KCl. KCl stimulates transcription of T4 DNA at concentrations above 0.1 M with an optimum 7 to 8 fold stimulation observed at concentrations of 0.30 M to 0.31 M. Class 2 is composed of divalent cations represented by Mg^{++}, putrescine [$H_2N-(CH_2)_4-NH_2$], and cadaverine [$H_2N-(CH_2)_5-NH_2$]. These cations effect a 6 to 12 fold stimulation of RNA synthesis at concentrations ranging from 50 to 100 mM. Class 3 is represented by the polyamines spermine [$H_2N-(CH_2)_3-NH-(CH_2)_4-NH-(CH_2)_3$] and spermidine [$H_2N-(CH_2)_4-NH-(CH_2)_3-NH_2$] which stimulate RNA synthesis 2 to 3 fold at concentrations of 0.25 mM and 2.5 mM respectively. Class 2 and Class 3 compounds

are also stimulatory by 2 to 3 fold at optimal KCl concentrations. However, a Class 3 compound, e.g., spermidine, is not stimulatory at optimal concentrations of a Class 2 compound, e.g., $MgCl_2$, in the presence of or absence of KCl.

Difficulty is encountered in establishing the optimum polyamine concentration at low ionic strength because of template precipitation caused by low concentrations of polyamines. Moderate concentrations (0.05 to 0.1 M) of KCl, which have little effect on the in vitro transcription reaction, prevent precipitation of DNA by polyamines. Spermidine in the presence of a moderate concentration of KCl markedly stimulates transcription of T4 DNA. It is significant that millimolar quantities of polyamines elicit large stimulatory responses at moderate (0.1 M) and optimum (0.3 M) concentrations of KCl.

The stimulatory response elicited by cations is not universal with respect to the DNA template. Both KCl and spermidine, in the presence of 0.1 M KCl, greatly stimulate transcription of T4 and T7 DNA, but stimulate transcription of E. coli DNA, calf thymus DNA, and poly [d(A-T)] only slightly. Spermidine, in the absence of KCl, moderately stimulates transcription of all five templates.

Reinitiation by the RNA polymerase does not occur at low ionic strength resulting in early cessation and plateauing of RNA synthesis. Early plateauing is not observed when stimulatory concentrations of KCl or spermidine are present since their presence allows reinitiation.

Polyamine-mediated stimulation cannot be attributed simply to the polyamine contribution to the net ionic strength since, in all cases, their presence contributes considerably more to an increase in synthetic activity than to the net ionic strength.

Analysis of reaction mixtures containing ^{32}P-labeled T4 DNA by sedimentation in sucrose gradients and filtration on nitrocellulose membrane filters revealed that nascent RNA is released at low ionic strength in the presence or absence of spermidine. Thus, facilitation of nascent RNA release from the transcription complex, i.e., RNA-Enzyme-DNA, is not responsible for polyamine-mediated stimulation of *in vitro* RNA synthesis.

Neither spermidine nor KCl were found to influence the size of *in vitro* transcripts synthesized on T4 DNA. Analysis of product RNA was performed by sedimentation in sodium dodecyl sulphate-sucrose gradients and formaldehyde-sucrose gradients.

Both KCl and spermidine were found to be effective in allowing dissociation of the RNA polymerase from the template on which it originally initiated and reinitiation of new RNA chain synthesis on a second or "challenger" template. Transcription of the "challenger" template does not occur at low ionic strength in the absence of spermidine.

Restart of RNA synthesis once the plateau phase had been established was effected by both 0.3 M KCl and 6 mM spermidine + 0.1 M KCl. Spermidine added to the stalled reaction in the absence of KCl caused precipitation in the system.

Spermidine was found to be somewhat more effective in preventing and overcoming inhibition of RNA synthesis by addition of either *E. coli* tRNA (stripped) or 16 S and 23 S ribosomal RNA.

INTRODUCTION

Ever since Herbst and Snell (1) first demonstrated that Hemophilus parainfluenzae requires polyamines for growth, a large body of literature has accumulated relating polyamines with vital life process. The diamine putrescine, and the polyamines spermine and spermidine, principally spermidine, have been implicated as playing important roles in the control of RNA synthesis in both prokaryotic and eukaryotic organisms. The majority of this literature contains data relating, in a correlative manner, polyamine synthesis and accumulation with that of RNA. However, recent data frequently implies a direct cause and effect relationship between polyamine metabolism and RNA synthesis (2-7). This subject has been adequately reviewed in several recent articles (8-10), symposia (11-12), and extensively in a recent book (13).

Interested by reports of in vivo studies implicating polyamines with control of RNA synthesis, several investigators undertook studies involving the direct effect of polyamines on purified DNA-dependent RNA polymerases from microbial sources. Before any review dealing with polyamines and in vitro RNA synthesis is initiated, a brief discussion of the in vitro RNA synthetic reaction is in order.

Escherichia coli DNA-dependent RNA polymerase is a high molecular weight protein composed of several subunits. Burgess (14) and Zillig et al. (15) have independently reported that the polymerase consists of three subunits α, β, and β' with the respective molecular weights of 40,000, 150,000 and 160,000. These subunits in a molar ratio of 2 α: β:β' form what is referred to as the core enzyme. A fourth subunit,

sigma, with a molecular weight of 80,000, as reported by Burgess *et al.* (16) and Berg *et al.* (17), combines with one molecule of core enzyme to form the so-called holoenzyme. Sigma is separated from the core enzyme by chromatography on phosphocellulose (16). Most existing methods of preparation of RNA polymerase give a mixture of core enzyme and holoenzyme (25).

The RNA polymerase holoenzyme is a monomer at high ionic strength with a sedimentation constant of 15 S while exhibiting a sedimentation constant of 23 S at low ionic strength due to dimerization (18). The significance of the monomeric and dimeric forms is, as yet, unclear. However, it has been suggested that the monomer is the catalytic unit (51).

A subunit of 10,000 molecular weight, designated ω, has also been isolated in many preparations of bacterial RNA polymerases. However, it is unclear whether ω is a normal constituent of the holoenzyme or a tightly bound impurity since Burgess (14) has reported RNA polymerase preparations lacking ω which exhibit normal activity.

Purified RNA polymerases from other bacterial sources appear to be similar in molecular weight and basic subunit composition to the *E. coli* RNA polymerase (19-22).

The *in vitro* RNA synthetic reaction can be conveniently described as consisting of the following events: association, initiation, polymerization or chain elongation, and termination. The association event consists of the binding of RNA polymerase to the DNA template resulting in the formation of a DNA-enzyme complex. The events leading to the binding and condensation of the first two ribonucleoside triphosphates with the formation of the first nucleotide bond are referred to as

initiation. Once initiation has been effected, ribonucleoside triphosphates may be incorporated into the growing polyribonucleotide chain with the release of pyrophosphate. This process is known as polymerization or chain elongation. Eventually, after an appropriate elongation period, a point is reached at which synthesis must be terminated. Release of the nascent RNA chain and reinitiation by the polymerase constitute termination.

With this brief discussion of the reaction mechanism, a more detailed discussion and review of each reaction event, the function of particular subunits in that event, and the effect of polyamines and inorganic cations on these events will follow. It is, however, not the intent of this review to discuss in great detail the individual reaction events mentioned above. Therefore, discussion will be limited to include only that information which allows a clear and concise review of the role and/or effects of polyamines and inorganic cations in and on these events. Several review articles have recently been published which give detailed discussions of all aspects of DNA-dependent RNA polymerase (23-35).

Association

RNA polymerase has been shown, by several different methods, to bind to DNA in the absence of nucleoside triphosphates. Fox et al. (36) showed that RNA polymerase from Micrococcus lysodeikticus binds to calf thymus DNA forming a complex sufficiently stable to allow separation of the enzyme-DNA complex from the free polymerase by centrifugation on a glycerol gradient.

Jones and Berg (37) developed a nitrocellulose membrane filter technique to study polynucleotide-RNA polymerase interaction. They reported

that native DNA and RNA polymerase separately pass through Millipore membrane filters while a complex of the two are retained quantitatively by the filter. With this method they measured the stoichiometry of the binding of E. coli RNA polymerase to T7 DNA. Interpretation of data obtained by this method has been challenged recently by Hinkle and Chamberlin (38). They reported that RNA polymerase binds to the membrane filter even without prior association with DNA and, furthermore, a DNA molecule is retained by the filter if only one polymerase molecule is complexed with it.

The association event appears to be rapid since Richardson (39) reported that T4 DNA - E. coli RNA polymerase complexes are detectable, with the membrane filter method, within 15 seconds of the addition of the enzyme to the DNA solution.

The reaction appears to be reversible since Richardson (39) also showed that once bound to one DNA template, the polymerase can dissociate and bind another template. In addition, Sternberger and Stevens (40) have reported that labeled polymerase will compete for sites with unlabeled polymerase already bound to a DNA molecule. However, 1/6 of the sites appeared to be bound tightly enough so as to be unavailable for competition.

Other investigators have reported two classes of binding sites (41-42): a large number of weak reversible binding sites and a smaller fraction of stable irreversible binding sites.

The experiments mentioned above were conducted with polymerase preparations containing undetermined amounts of core and holoenzyme. Very recent data (38) reported by Hinkle and Chamberlin using preparations of core and holoenzyme from E. coli showed that core enzyme binds

to a large number of sites on T7 DNA but forms only weak complexes. Interaction of RNA polymerase holoenzyme with T7 DNA, however, leads to two classes of complexes: a small number of stable complexes and a larger number of weak complexes. They concluded that sigma reduces the affinity of RNA polymerase for random regions of DNA but enhances binding of the enzyme to a selected number of sites, presumably corresponding to promoter sites. They also reported that the formation of stable holoenzyme-DNA complexes was strongly affected by temperature, proposing that the formation of these complexes requires the opening or melting of the DNA strands in the region of the binding site, and that this task is accomplished by sigma.

Zillig, et al. (43) have been able to dissociate the E. coli RNA polymerase into subunits and selectively reconstitute active enzyme molecules using a dissociation procedure involving large molar concentrations of LiCl. They found that an α-free ββ' complex caused retention of labeled T4 DNA by membrane filters while α essentially lacked this ability. β' was also found to be the only subunit which reacted with heparin, a strong competitor of DNA for association with the polymerase. Sethi (24) also reported that β' associated with DNA effectively while β and α did not. These results have been the basis for the designation of β' as the DNA binding subunit.

RNA polymerase is also able to bind RNA. Several investigators have shown that RNA is an effective inhibitor of DNA-directed RNA synthesis (36, 44-48). Fox et al. (36) using M. lysodeikticus RNA polymerase showed that E. coli ribosomal RNA, MS2 viral RNA, calf thymus cRNA, and polyuridylic acid (poly U) were effective inhibitors of the polymerase reaction. However, they pointed out that the order of addition of the com-

ponents was important. Inhibition was found to be effective only if the enzyme was preincubated with the RNA indicating that there exists a competition between RNA and DNA for a site on RNA polymerase. As with the DNA-enzyme complex, they were also able to show 4S RNA-enzyme association on glycerol gradients.

Similar data concerning inhibition by addition of exogenous RNA has been reported for the E. coli RNA polymerase (47, 46, 48, 39, 51) and for RNA polymerase from Azotobactor vinelandii (45).

Anthony et al. (48) have reported that there is an ordered preference of binding of nucleic acid polymers by the polymerase. They demonstrated a displacement of one polymer from the enzyme by a second polymer. The order of preference of binding was found to be: single-stranded sonicated DNA $\geq$ double-stranded sonicated DNA > single-stranded DNA > tRNA > native DNA. Therefore, tRNA was actually able to displace native DNA from the polymerase. However, once initiation had occurred the DNA-enzyme complex was stable against dissociation by tRNA.

It appears, then, that RNA as well as DNA binds to purified RNA polymerase from E. coli, M. lysodeikticus and A. vinelandii. Various species of RNA also inhibit DNA-directed synthesis of RNA by competing with DNA for a site on the polymerase and this competition is no longer observed once initiation has been effected.

The salt concentration has been reported to have a dramatic effect on the degree of nucleic acid-enzyme association. Anthony et al. (48) reported that 0.4 M $(NH_4)_2SO_4$ added to an RNA polymerase reaction mixture prior to addition of enzyme resulted in almost complete inhibition of RNA synthesis. They showed directly, by use of the membrane filter method, that as the concentration of $(NH_4)_2SO_4$ increased, the amount of

labeled DNA complexed by the enzyme decreased. A concentration of 0.266 M $(NH_4)_2SO_4$ was found to effectively prevent retention of the labeled DNA by the filter. 0.266 M KCl was also able to dissociate the DNA-enzyme complex.

Richardson (39) obtained similar data with 0.5 M KCl. In addition, he found that the transcription complex, i.e., enzyme-DNA-RNA, was stable during sedimentation on sucrose gradients containing 0.5 M KCl whereas the DNA-enzyme complexes formed prior to initiation were not.

In addition to observing dissociation of DNA-enzyme complexes at high ionic strength, Anthony et al. (49) reported similar results with RNA-enzyme complexes. It was later confirmed (48) that in sucrose gradients containing 0.022, 0.044, and 0.066 M $(NH_4)_2SO_4$; 52, 66, and 90% of the RNA-enzyme complexes were dissociated respectively. Similar results were observed with NH_4Cl.

A comparison of the membrane filter and sucrose gradient methods showed that salt appeared less effective in dissociating nucleic acid - polymerase complexes by the filter method than by the gradient method, presumably because of the influence of the centrifugal field.

The inhibition by RNA of DNA-directed RNA synthesis mentioned previously was reported to be prevented by high ionic strength by Fox et al. (36) using the M. lysodeikticus enzyme. So et al. (51) reported similar results with 0.2 M KCl using the E. coli RNA polymerase and T4 DNA as template.

Divalent cations are not necessary for nucleic acid-polymerase association. Fox et al. (36) were able to obtain both RNA and DNA complexes with M. lysodeikticus RNA polymerase without the addition of divalent cations. Likewise, Richardson (39) using the E. coli polymerase showed

that omission of Mg^{++}, which was required for RNA synthesis, and Mn^{++}, which inhibited RNA synthesis by 20%, had no effect on DNA-enzyme association. However, removal of Mg^{++} with EDTA stopped polymerization instantly. Anthony et al. (48) reported 24% less DNA bound to the same amount of enzyme when Mg^{++} and Mn^{++} were removed with 10^{-3} M EDTA. Similar results were reported by Sternberge and Stevens (40).

It appears, then, that divalent cations are not required for association of nucleic acids with RNA polymerase and, in fact, may slightly interfere with their interaction.

Polyamines have been reported to have differential effects on nucleic acid-RNA polymerase complexing. Richardson (39) studied the E. coli enzyme and was able to observe no difference in DNA-enzyme association with 0.4 mM spermine present. Fox et al. (36) used the M. lysodeikticus enzyme and showed that the DNA-enzyme complexes, as analyzed by glycerol gradient analysis, were not affected by the addition of spermidine to the gradient buffer. However, MS2 RNA-enzyme complexes, analyzed by the same method were completely destroyed by 2 mM spermidine in the gradient. In addition, they were able to partially prevent inhibition by exogenous RNA by having 2 mM spermidine present in the reaction mixture.

Krakow (50) showed reversal of inhibition of the A. vinelandii enzyme by polyfluorouridylic acid, a reported competitor of DNA for the primer site on the polymerase (45), with 12 mM spermidine present in the reaction mixture. The capacity of polyamines to prevent inhibition by exogenous RNA has also been shown with E. coli RNA polymerase (91).

Thus polyamines appear to be very effective at low concentrations in dissociating RNA-enzyme complexes but have little effect on DNA-enzyme

complexes.

Initiation

Initiation has been defined by Anthony et al. (52) as the addition of the 5'-terminal and the subterminal nucleoside triphosphates to the DNA-enzyme complex with the subsequent formation of the first phosphodiester bond. That the direction of synthesis is from the 5' to the 3' terminus was earlier reported by Maitra and Hurwitz (59) and Bremer et al. (60).

The complexity of the DNA-directed RNA synthetic reaction has hampered the construction of models. Goldthwait et al. (53), however, have proposed a simple model consistent with many of the observations based on studies of reaction kinetics (52), equilibrium dialysis (54), and fluoresence quenching (55).

The model proposes the existence of two nucleoside triphosphate binding sites on the RNA polymerase. The first site binds purines but not pyrimidines. This is based on the stablization of DNA-enzyme complexes by purines, but not pyrimidines, in the presence of high salt. This site is designated the initiation site since it possesses a dissociation constant equivalent to the Km of initiation and is susceptible to inhibition by rifamycin, a specific inhibitor of initiation (56, 57, 58). Initiation by purines as the 5'-terminal nucleoside has been confirmed in a direct manner by the isolation of purine nucleoside tetraphosphates after alkaline hydrolysis of RNA synthesized in vitro (59, 60). Similar analysis of in vivo RNA also reveals purines as the predominant 5'-terminal nucleoside (61). Binding of the nucleoside

triphosphate to this site occurs in the presence or absence of divalent cations (54).

The second site, designated the polymerization site, requires Mg^{++}, is not sensitive to rifamycin, and shows no selectivity for purines or pyrimidines. More will be said about the divalent cation requirement later.

This model is based, in part, on the observation that the apparent Km for the 5'-terminal nucleoside triphosphate is 10 times larger than the Km for the ribonucleoside triphosphates incorporated into the rest of the RNA chain. Downey and So (65), however, report no difference in Km values.

Krakow (19, 62) has also proposed a model for DNA-directed synthesis of RNA. Two sites are proposed in this model as well: an initiation site with a strong preference for purines and a substrate binding site where the incoming nucleoside triphosphate binds, and is, presumably, hydrogen bonded to the template. The two nucleoside triphosphates are joined by the formation of a phosphoester bond and subsequent release of pyrophosphate. A translocation step follows with the initiation site then becoming the product terminus site and the substrate site becoming free to accept another nucleoside triphosphate. Although requirements for divalent cations are not discussed in this model, all experiments contained Mg^{++}.

Exactly at what point the DNA-enzyme complex becomes stable to high salt concentrations is still controversial. Di Mauro et al. (63) reported that RNA polymerase - T4 DNA complexes were stable in the presence of KCl up to 0.3 M once the first nucleoside triphosphate, a purine, was incorporated into the complex. The claim that only one

nucleoside triphosphate is required was based on the assumption that absolutely no RNA is synthesized in their system when UTP is lacking since UTP is reported to be the second nucleoside triphosphate incorporated in normal T4 RNA synthesis.

Similar data showing stabilization of the complex by purines was reported by Anthony et al. (64). However, the exact nature of the DNA-enzyme-purine complex was not determined. Sentenac et al. (92) reported that with poly d(AT) as template, the presence of one nucleoside triphosphate (ATP) did not result in a stabilization of the enzyme-DNA complex. Chamberlin (25) has commented on the apparent inability of a single nucleoside triphosphate to confer stability on DNA-enzyme complexes at high salt as reported by several laboratories.

The sigma subunit or factor has been found to have a profound effect on initiation. Burgess et al. (16), who first reported the separation of sigma and the core enzyme by phosphocellulose chromatography, found that core enzyme exhibited little activity with a T4 DNA template, whereas, the addition of sigma increased activity over 60-fold.

Transcription was reported to be stimulated somewhat less by sigma with T7, λ and E. coli DNA templates and very little stimulation was observed with calf thymus DNA, denatured DNA, or poly d(AT) (33). The reason for lower stimulation with the last three templates is that core enzyme transcribes these templates efficiently in the absence of sigma.

Travers and Burgess (66) soon showed that the increased transcription in the presence of sigma is due to an increase in the number of chains initiated and not due to an increased rate of chain growth. Furthermore, they showed that sigma is released after initiation and is then available for re-use by other core enzymes.

Based on these first experiments, evidence has steadily accumulated giving sigma the role of determining initiation specificity. As mentioned previously, Hinkle and Chamberlin (38) reported that sigma affects DNA-enzyme associations by destabilizing non-specific binding sites and stabilizing specific binding sites, presumably promoter sites. That sigma is selective for actual promoter sites is shown by the fact that in vitro RNA synthesis in the presence of sigma on T4 DNA is restricted to the initiation sites utilized in vivo (67, 68). Similar data has been reported for T7 DNA (69, 70).

Sugiura et al. (76) have shown that sigma affects the asymmetry of transcription. Core polymerase was found to transcribe both the plus and minus strands of fd phage replicative form DNA while holoenzyme transcribed only the minus or biologically correct strand.

Sigma may not, however, contain all the information needed for recognization of the promoter site. There is evidence that the β subunit is also involved in the initiation process since rifampicin appears to bind to the β subunit (19) and does not attack sigma (63). Furthermore, rifampicin-resistant bacterial mutants have been shown to carry the resistance phenotype in the β subunit (71). β also seems to be involved with sigma function (19) since sigma does not bind to DNA (15, 72) but is reported to bind to the β subunit (72).

As mentioned earlier, Travers and Burgess (66) reported that sigma is released after initiation. Krakow and Von der Helm (19) have shown by use of acrylamide gel electrophoresis that A. vinelandii sigma factor is released some time after initiation and that the release of sigma appears to require synthesis of a small polyribonucleotide of undetermined length. Ruet et al. (73) have reported similar data with

E. coli polymerase and Gerade et al. (74) with the polymerase from Pseudomonas putide.

Krakow (75) also reported the release of sigma from A. vinelandii polymerase after association with tRNA or Poly U. In fact, incubation of RNA-polymerase holoenzyme with single stranded polyribonucleotide or polydeoxribonucleotides resulted in release of sigma, whereas sigma release occurs in the presence of double stranded polydeoxyribopolymers only after synthesis of some polyribonucleotides.

Salt seems to effect initiation predominantly in a negative manner. As previously discussed, high salt concentrations result in a dissociation of enzyme-DNA complexes. This shows up in the reaction kinetics as a lag phase under certain conditions. Walter et al. (77) reported that synthesis in the first few minutes of incubation is reduced when the temperature is reduced at constant ionic strength or the ionic strength increased at constant temperature. This lag increased rapidly below a temperature of 17°C or at 37°C at salt concentrations above 0.04 M $MgCl_2$ or 0.30 M NH_4Cl (78), and is reduced by preincubation of the enzyme with DNA. No lag is observed with single stranded DNA. It was concluded that the lag phase was due to a required localized melting of the DNA at the initiation site before synthesis began and that salt influenced this process by raising the energy of activation required for the enzyme to melt part of the DNA double strand. The effect of salt at the association level was thought not to be responsible for the observed decrease rate of initiation. The occurence of a lag phase at high concentrations of both mono and divalent salts has been reported by others (78-80).

Although So et al. (51) did not report the occurence of a lag phase at high salt concentrations, they suggested that an observed increase in initial rate of synthesis in the presence of 0.2 M KCl was due to the observed dissociation of the polymerase from a dimeric to a monomeric form at high ionic strength (18). They also suggested that KCl decreases the affinity of RNA polymerase for non-specific binding sites on the template since KCl inhibits polyadenylic acid (poly A) synthesis with either T4 DNA or poly U as template.

Salt has been reported to influence other aspects of initiation. Maitra and Barash (81) reported that KCl increased the number of RNA chains initiated with ATP and decreased the number starting with GTP with T4 DNA as template. Zillig et al. (43) have reported that in the absence of template, sigma was required to bind purine ribonucleoside triphosphate to the enzyme. At high salt concentrations, however, sigma was not required. Pyrimidines were not bound in either case.

This raises the question as to whether sigma is necessary or functions at all at high salt concentrations. Maitra et al. (81) reported that transcription products of T4 DNA were copied by the holoenzyme exclusively from the ℓ strand and appeared to be the same as the in vivo products in the presence or absence of 0.2 M KCl. Also no stimulation of RNA synthesis by high salt is observed with core enzyme (82, 84). Therefore, salt does not appear to interfere with the action of or replace sigma.

As mentioned previously, Mg^{++} is required for RNA synthesis as evidenced by the immediate cessation of polymerization when Mg^{++} is removed (50, 78). That Mg^{++} is required for initiation is suggested by Anthony et al. (54). As mentioned, they proposed two binding sites for

nucleoside triphosphates, the second or polymerization site requiring divalent cations. The co-factor role of Mg^{++} for E. coli polymerase appears, then, to be associated with the polymerization site and is needed during initiation for binding of sub-terminal nucleoside triphosphate and during elongation for binding of incoming nucleoside triphosphates. Mn^{++} appears to play a similar role for the A. vinelandii polymerase (83). Fuchs et al. (78) have reported that for the E. coli RNA polymerase Co^{++} and Mn^{++} can replace Mg^{++} in this co-factor role.

Whether polyamines can replace inorganic divalent cations in this co-factor role has not been determined directly. However, it seems unlikely since Krakow (50) with the A. vinelandii enzyme and Fuchs et al. (78) with the E. coli polymerase have shown that no synthesis is allowed in the absence of divalent cations, even in the presence of polyamines.

Polyamines do appear to have some effect on initiation since Gumport (93) showed that spermidine promotes asymmetric transcription by the M. lyxodeikticus polymerase on ϕX174 replicative form DNA. Mg^{++}, and to a lesser degree KCl, also increased asymmetric transcription in the system.

The mechanism underlying the promotion of asymmetric transcription by spermidine is unclear since both spermidine and spermine have been shown to have little effect on DNA-enzyme association (39, 36). Abraham (90) suggested that polyamines may have an effect on initiation by influencing the secondary structure of defective single stranded regions of DNA, thereby reducing non-specific binding of the enzyme to these defective regions and thus making more enzyme molecules available for association with the initiation sites. No direct evidence for this

model, however, has been presented.

Petersen et al. (91) using calf thymus DNA as template and E. coli RNA polymerase reported that more RNA chains were initiated in the presence of spermidine than in its absence. Although they did look at both ^{14}C-ATP and γ-^{32}P-ATP incorporation, they reported the increased incorporation of both isotopes sufficiently late in the assay that some reinitiation could have taken place. Therefore, the reported increase in the number of chains could be a representation of both initiation and reinitiation events. Synthesis was not increased by spermidine when denatured DNA was used as template.

The effects of polyamine on core enzyme have not been tested. Therefore, it is unknown whether spermidine stimulates RNA synthesis in the absence of sigma.

Chain Elongation

Chain elongation can be defined as a series of undetermined repetitious events involved in the addition of nucleoside residues to the nascent RNA chain by linkage of the ribose 3'-hydroxyl to the 5'-phosphate group of the incoming nucleoside triphosphate, release of inorganic pyrophosphate (PPi), and subsequent translocation along the template.

Little is known about the actual chemistry of the polymerization reaction, nevertheless, two models have been proposed (85, 86) which are in complete disagreement with each other. A discussion of these models is not relevant to this review.

The chain elongation step does have a specific inhibitor, streptolydigin. It has been proposed by Cassani et al. (88) that streptolydigin inhibits chain elongation by affecting the rate of phosphodiester bond

formation. However, no mechanism was presented. Although the entire core enzyme is required for polymerization (43), the β subunit is more directly implicated since mutations giving streptolydigin resistance are found to map on the same gene as rifampicin resistance (87).

Early studies of chain growth using a T4 DNA template at 37°C suggested that the in vitro rate of elongation was much lower than that observed in vivo. Bremer and Konrad (94) reported a rate of 2 to 3 nucleosides polymerized/second for in vitro T4 DNA transcription while Bremer and Yaun (95) reported a rate of 28 nucleosides/second for in vivo T4 RNA chain growth. In 1970, Richardson (96) using the formaldehyde-sucrose gradient technique of Boedtker (97) showed, by observing the size of the RNA chains produced with time, that the rate of elongation was dependent upon the ionic strength. This data has been confirmed by Bremer (80) using a similar method.

Witmer (89) and Maitra and Barash (82), using ATP and GTP labeled with ^{32}P in the γ position to determine chain length by comparing the total RNA made with the number of initiation events (^{3}H-NTP/^{32}P-ATP + ^{32}P-GTP), reported no influence of high ionic strength on the rate of chain elongation.

The reverse of polymerization, hydrolysis of the phosphodiester bond, also occurs during the polymerization reaction. Krakow and Fronk (62) detected PPi exchange once the dinucleoside was formed. Before the formation of the first phosphodiester bound, no PPi exchange was detected. Bautz et al. (67) reported that 0.2 M KCl is required to maximize PPi exchange but found that it also inhibits the addition of the second nucleoside to the initiation complex.

Although it is implied by observations of the overall reaction rate that polyamines stimulate chain elongation, direct documentation is lacking. Fox et al. (36), Abraham (90), Krakow (50), Petersen and Kroger (79) and others (18, 51, 44, 91) have reported stimulation of the overall reaction by polyamines. A few investigations showed some stimulation of the initial rate by spermidine (78, 44, 90). However, direct evidence of stimulation of the elongation rate by examination of the size of the product with respect to time (96, 80), examination of PPi exchange (67) or use of the γ-labeled nucleoside triphosphates method (83, 89) have not been reported. Therefore, it is questionable whether the observed stimulation of initial rate is due to an increased rate of initiation or chain elongation.

Petersen et al. (91) did examine incorporation of ^{14}C-ATP and γ-^{32}P-ATP using calf thymus DNA and E. coli RNA polymerase but reported only one time point showing a slight increase in the $^{14}C/^{32}P$ ratio at 20 minutes in the presence of spermidine.

Termination

The termination event which includes release of nascent RNA and reinitiation has been and still is a controversial subject.

Early studies of in vitro DNA transcription showed that under the usual conditions of the assay (low ionic strength) synthesis was linear for the first 10-15 minutes but then began leveling off and usually ceased by 60 minutes (98). Such a reaction was said to display "plateau" kinetics.*

* The term "plateau" kinetics was introduced by Fuchs et al. (78) to describe the early reduction and cessation of RNA synthetic activity observed at low ionic strength.

Bremer and Konrad (94) reported that with E. coli RNA polymerase and ^{32}P-labeled T4 DNA template the newly synthesized RNA is not released from the transcription complex. When they placed an RNA polymerase reaction mixture on sucrose gradients, the labeled RNA and DNA were observed to co-sediment. This complex was dissociated with sodium dodecyl sulfate suggesting to them that the complex was held together by a protein, presumably the polymerase. They proposed that at low ionic strength conditions, RNA is not released, and a ternary complex of RNA-enzyme-DNA is formed.

Krakow (99) reported similar results with the A. vinelandii polymerase and was able to demonstrate stimulation of RNA synthesis by the introduction of RNase, following the rate of transcription by observing ^{32}PPi release from γ-^{32}P-labeled ribonucleoside triphosphates.

These reports, in addition to the many reports of inhibition of RNA synthesis effected by the addition of exogenous RNA (36, 44-48) resulted in a generally accepted proposal that cessation of synthetic activity observed at low ionic strength was due to product inhibition by the nascent RNA with the formation of a ternary complex of DNA-enzyme-RNA.

Fuchs et al. (78) in 1967 showed that initiation and reinitiation appeared to occur for several hours at an ionic strength of 0.36. That is, early plateauing did not occur in the presence of high salt. This observation has been confirmed (51, 59, 82).

In 1969, Maitra and Barash (82) reported, by following the incorporation of γ-^{32}P-labeled ATP, that reinitiation does occur at high ionic strength but not at low ionic strength. Furthermore, they showed with sucrose gradients that the nascent RNA is released when synthesized

under high salt conditions. They also reported some release of RNA under low salt conditions in contradiction to the report of Bremer and Konrad.

Richardson (23), in 1969, reported similar results by use of the membrane filter method. He found that the transcription complex was retained by the filter while free RNA was not and that, by a comparison of the total RNA synthesized with the amount of complex bound RNA remaining on the filter, an estimate of the amount of RNA released could be determined. He also observed some release of RNA at low ionic strength at later times in the reaction.

In 1970, Millette and Trotter (100) reported that although reinitiation did not occur at low ionic strength, release of nascent RNA did. Release of RNA was observed by use of sucrose gradients, in both low and high salt assays.

Early in 1971, Witmer (89), using both the membrane filter and sucrose "bulk" gradient methods, reported that RNA was released at high salt conditions but that absolutely no RNA was released at low ionic strength reconfirming the findings of Bremer and Konrad.

Slightly later in 1971, Jenkins et al. (101) reported that with T7 DNA and E. coli RNA polymerase, newly synthesized RNA is released at both salt conditions. Release of RNA at low ionic strength has recently been confirmed by Goldberg and Hurwitz with T4 DNA (102).

There has also been considerable interest in the effect of ionic strength on characteristics such as product RNA size and fidelity to in vivo synthesis. Millette and Trotter (100) using T4 DNA reported product RNA ranging from 25 S to 44 S with a number average chain length of 5,000 to 7,500 nucleosides and a weight average chain length of 11,500

nucleosides under both conditions. Witmer (89) using the same T4 system reported two discrete size classes of RNA. 70% of the total RNA was 4,300 nucleosides long while the remainder was reported to be 7,100 nucleosides long. That there is no difference in the size of T4 or T7 DNA transcription products synthesized under high or low salt conditions has been confirmed by others (103, 81).

Petersen and Kroger (79) using E. coli RNA polymerase and calf thymus DNA did, however, find a larger product formed in the presence of 0.32 M NaCl. Also, Richardson (104) reported that in vitro T4 product RNA was 1.3 times longer when synthesized at high salt conditions.

Qasba and Zillig (105) showed in 1968 by competition hybridization studies, using the T4 system, that the RNA synthesized in vitro at both low and high ionic strength was composed of only early RNA species. That is, by competition hybridization with mRNA from cells infected with T4 phage, the in vitro RNA made under both salt conditions contained RNA which competed with the in vivo RNA transcribed early (1-5 min. after infection) and not the in vivo RNA transcribed late (30-32 min. after infection).

Maitra et al. (81) have confirmed the data of Qasba and Zillig. In addition they showed with both T4 and T7 DNA that the in vitro product terminates predominantly with uridine as the 3'-terminal nucleoside indicating that the polymerase probably recognizes specific termination sites on the DNA. Furthermore, they showed that the high salt products appear to be physiologically significant since they served as highly effective messengers in cell-free amino acid incorporating systems. In fact, the T4 product directed the in vitro synthesis of active lysozyme, a gene known to be transcribed early in the infection cycle. Millette et al.

(103) have confirmed much of this work.

It appears then, that termination occurs at specific sites at both high and low salt conditions. The majority of the data indicates that the RNA chain is released at both salt conditions but reinitiation is allowed only at high ionic strength.

An appropriate question to ask at this point is, does the polymerase dissociate from the template it first initiates on when allowed to reinitiate a new RNA chain? Richardson (104) has shown by hybridization analysis of product RNA that when E. coli polymerase is allowed to initiate on T4 DNA, and T5 DNA is then added after 10 minutes under low ionic strength conditions, no product hybridizes to T5 DNA. However, when the same experiment is conducted at high salt conditions, a considerable portion of the product RNA hybridizes to T5 DNA. This indicates that the enzyme does indeed dissociate from the template when reinitiation of new RNA chains occur. Similar data using T4 and T7 DNA has been reported by Maitra et al. (81).

It is unclear in all of these studies exactly where the polymerase is located once the plateau is reached. Although it is assumed (102) that the polymerase remains with the template, no real documentation exists. If, indeed, RNA is released at low ionic strength conditions, the polymerase could easily be complexed with the RNA. This view is supported by RNA-enzyme binding studies (36), ability of exogenous RNA to inhibit RNA synthesis (36, 44-48) and stimulation of RNA polymerase activity by RNase (100).

In 1969, Roberts (106) reported the isolation of a protein factor of approximately 200,000 molecular weight which caused termination and release of RNA molecules in an in vitro reaction using bacteriaphage

λ DNA as a template. Although this factor, designated rho, was found to have no effect on initiation, its presence did result in decreased synthesis of RNA. Roberts found that this decrease was due to early termination. RNA synthesized in the absence of rho exhibited a broad distribution of size from 35 S to less than 5 S while RNA synthesized in the presence of rho was composed of two discrete species of 7 S and 12 S.

Roberts also reported that the presence of rho caused release of RNA from the transcription complex. This experiment consisted of comparing sucrose gradient profiles of reaction mixtures with and without rho after 20 minutes of incubation. Because of the difference in size of RNA made in the presence and absence of rho one would expect, if release of RNA occurred in both cases, to see a much greater proportion of free RNA at earlier times in the case where rho was present. It would have been of interest to see the gradient profile at 60 minutes of incubation.

Rho also appeared to increase the overall accuracy of transcription but had no effect upon the choice of promoter site.

In vivo transcription of T4 DNA prior to replication results in the production of two readily distinguishable species of RNA, immediate early and delayed early mRNA (107). The in vivo synthesis of delayed early RNA requires protein synthesis since treatment with chloramphenicol allows only synthesis of immediate early RNA (108). As mentioned previously, the in vitro transcripts of T4 DNA using E. coli RNA polymerase are composed of both immediate and delayed early RNA (105, 81). Also there is evidence that the immediate and delayed early RNA is synthesized as a continuous in vitro transcript (109). Richardson (110) showed that

RNA synthesized *in vitro* in the presence of rho contained only RNA species found in T4 RNA synthesized in chloramphenicol treated cells, i.e., immediately early RNA. This indicates that rho may, indeed, play a role *in vivo*.

The mechanism of rho-mediated termination is not clear. Richardson (110) reported that rho activity is insensitive to changes in DNA concentrations and only slightly sensitive to changes in enzyme concentration. He also conducted binding studies and found rho to bind to both DNA and RNA. However, RNA-rho interaction was found to be much stronger than DNA-rho association.

Davis and Hyman (111) using electron microscopy, reported that for T7M DNA, which is reported to have only one promoter site, RNA synthesis in the presence of rho proceeds from the promoter site along the template with the release of discrete RNA chains. The RNA polymerase under these conditions was observed not to be released.

Richardson (110) and others (81, 102) have reported that rho is not functional at ionic strength above 0.1.

Thus, two types of termination events appear to exist. One results in the release of discrete RNA chains but not of the polymerase, is mediated by rho and is not functional at high ionic strength conditions. The other results in release of RNA and, at high salt conditions, also of the polymerase. These observations have led Maitra *et al.* (81) to propose the existence of a hypothetical RNA termination site and an enzyme release site. According to this model, after initiation, RNA is released by the action of rho as the polymerase passes the RNA termination site but will not dissociate itself until the enzyme release site is reached.

A major problem yet to be resolved is the apparent inability of rho to function at high ionic strength. It is puzzling that under conditions most favorable for the RNA polymerase reaction which allows release of the polymerase and reinitiation, rho is unable to function. To date, no set of conditions exist using the T4 and T7 *in vitro* systems where both rho-mediated termination and reinitiation can take place.

Polyamines have been shown to prevent early cessation of RNA synthesis. Krakow (50) reported stimulation of RNA synthesis by polyamines in a time course experiment indicating that reinitiation was occurring. However, there appeared to be considerable nuclease contamination in his enzyme preparation. A comparison of the stimulation exerted by spermine, spermidine, and putrescine, revealed the optimum concentrations of the three amines to be 2 mM, 20 mM and 40 mM respectively. Stimulation was not observed with denatured DNA.

Fox and Weiss (44) reported that polyamines increase the rate and extent of RNA synthesis again showing that the optimum concentration of the polyamines increased in the order spermine, spermidine, putrescine, cadaverine. They also reported that when spermine was added to the reaction mixture 60 minutes after the start of the reaction, synthesis increased.

Fuchs *et al.* (78) showed, in a more convincing manner, that polyamines are able to prevent early plateauing. In their system which included T4 DNA as template and *E. coli* RNA polymerase, 8 mM spermidine was shown to allow RNA synthesis to proceed in a near linear manner for 120 minutes at a moderate ionic strength (0.13 M NH_4Cl). They also showed that 8 mM spermidine, at the same salt conditions, added at 90

minutes, restarted transcription releasing the plateau.

Petersen, et al. (91) using calf thymus DNA template and E. coli RNA polymerase, examined the RNA polymerase reaction mixture on sucrose gradients in an experiment similar to that performed by Bremer and Konrad (95). They found that the apparent transcription complex appeared to sediment faster when the incubation was carried out in the presence of spermidine. However, because the template was ill defined and was not labeled, this data is very difficult to interpret. They also followed γ-^{32}P-ATP incorporation to determine the amount of reinitiation. However, the reaction was continued for only 20 minutes.

In a more recent paper (79) these investigators have shown that initiation does continue over at least a 60 minute period with spermidine present but ceases at approximately 15 minutes in its absence at low ionic strength. They also showed by sedimentation analysis that there was no difference in the size of RNA made in the absence or presence of spermidine. Again, the use of an ill defined template makes interpretation difficult.

It appears that the presence of polyamines in the RNA polymerase reaction mixtures allows reinitiation. However, experiments examining the effects of polyamines on release of nascent RNA from the transcription complex, the size of product RNA, or the ability of RNA polymerase to release from the template have either not been done or have been performed under conditions which make interpretation difficult. Experiments directly showing reinitiation in the presence of polyamines have not been performed. Also, although it is well documented that an increase in ionic strength does allow reinitiation to occur, a paper adequately

comparing the effects of polyamines and high ionic strength under similar conditions is lacking.

METHODS

RNA Polymerase Purification

DNA-dependent RNA polymerase (ribonucleoside triphosphate: RNA nucleotidyl transferase, EC 2. 7. 7. 6) was purified from Escherichia coli K-12 by the procedure of Burgess (112). 50 grams of frozen E. coli K-12 cells (Grain Processing Corporation, Muscatine, Iowa) were placed in an 8 oz. Osterizer blender container surrounded by ice. 125 gm of chilled glass beads (Superbrite 100, 3M Company, St. Paul, Minnesota) and 50 ml of cold Buffer G [0.05 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, 0.2 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, 5% (V/V) glycerol] were added. The cells were homogenized at low speed for 5 minutes and at high speed for 10 minutes. Grinding was followed by treatment with 4 µg per ml of pancreatic DNase (RNase-free, Worthington Biochemical Corp., Freehold, N.J.) for 30 minutes. This and all subsequent steps were performed at 4°. After the initial mixing upon addition of DNase, the glass beads were allowed to settle. The supernatant was decanted at the end of the DNase treatment and the beads were collected and washed with 25 ml of Buffer G in a large funnel loosely plugged with glass wool. The filtrate and supernatant were combined to yield Fraction I.

Fraction I was centrifuged at 78,480 x G (G average) for 2 hours at 4° in a Spinco No. 30 rotor to remove cell debris and ribosomes. The resulting supernatant (Fraction II) was brought to 33% saturation with $(NH_4)_2SO_4$. After 30 minutes of stirring, the solution was centrifuged at 6,000 x G in an International 858 rotor for 30 minutes. The 33% supernatant was brought to 50% saturation with $(NH_4)_2SO_4$ and treated as just described. The precipitate from this fraction contained the polymerase

and was resuspended in 260 ml of a 42% saturated ammonium sulfate solution in Buffer A (0.01 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol), stirred for 45 minutes, and centrifuged for 60 minutes at 6,000 x G as before. The pellet was dissolved in Buffer A and then diluted with Buffer A until the solution had a specific conductivity of 1.0×10^4 μmhos per cm at 4°. This solution was designated as Fraction III.

Fraction III was applied to a 25 ml volume Whatman microgranular DEAE-cellulose column with a length-width ratio of 5. The column was equalibrated with several column volumes of Buffer A prior to application of Fraction III and subsequently washed with 50 ml of Buffer A and 400 ml of Buffer A + 0.13 M KCl. The polymerase was then eluted with 300 ml of Buffer C + 0.23 M KCl (Buffer C = 0.05 Tris-HCl, pH 7.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol). The pooled active fractions constituted Fraction IV.

Fraction IV was precipated with 1.5 volumes of a saturated ammonium sulfate solution. The resulting precipitate was dissolved in the minimum volume of Buffer A to obtain complete dissolution and dialyzed against 100 volumes of Buffer A for 4 hours with one change of buffer. The dialyzed protein was diluted to a protein concentration of about 20 mg per ml and layered on a 10-30% glycerol gradient in Buffer A and centrifuged in a Beckman model-L2 65B ultracentrifuge at 75,465 x G for 24 hours at 4° in a Spinco SW 25.2 rotor. The active fractions from the gradient were pooled and again precipitated with 1.5 volumes of a saturated solution of ammonium sulfate, redissolved in Buffer A + 1.0 M KCl and dialyzed as before against Buffer A + 1.0 M KCl. This protein solution was layered on a 10-30% glycerol gradient in Buffer A + 1.0 M KCl and centrifuged for 30 hours at 75,465 x G at 4°. The active fractions

were pooled and prepared for storage by precipitating with 1.5 volumes of a saturated ammonium sulfate solution and dissolving the pellet in a storage buffer containing 0.01 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 0.1 M KCl, 0.1 mM dithiothreitol, 0.1 mM EDTA, and 50% glycerol. This solution was stored at=20° at a concentration of 5.0 mg per ml. The specific activity of the enzyme preparation was determined to be 1,250 units per mg protein using the assay prescribed by Burgess (112) where one activity unit of enzyme incorporates 1 mμ mole of AMP in 10 minutes.

Protein concentrations were determined on unpurified fractions by the procedure of Lowry (113). The protein concentration of purified RNA polymerase was determined by observing the optical density at 280 nm using an extinction coefficient of $E_{280}^{(1\%)} = 6.5$.

RNA Polymerase Assay

A normal low ionic strength assay ($\mu = 0.05$) contained 10 μmoles Tris-HCl, pH 7.9, 1 μmole $MgCl_2$; 200 mμ moles ATP, GTP, CTP; 100 mμ moles ^{3}H-UTP (7,500 dpm/mμ moles); 20 mμ moles dithiothreitol (DTT); 15 μg T4-DNA; and 2.5 μg RNA polymerase in a final volume of 0.2 ml. High ionic strength assays ($\mu = 0.35$) were identical to low ionic strength assays with the addition of 60 μmoles of KCl. Levels of spermidine (0.2 to 1.2 μmoles), spermine (0.02 to 1.2 μmoles), putrescine (2 to 20 μmoles), cadaverine (2 to 20 μmoles), and $MgCl_2$ (2 to 20 μmoles) were also tested for effects on the polymerase reaction. Assay tubes were incubated at 37° and 10 μl aliquots were removed at the appropriate times and spotted on 25 mm Whatman #40 filter paper discs which were subsequently air dried for 10 minutes before immersion in cold 10% trichloracetic acid (TCA). After soaking for at least 15 minutes the filters were washed as follows: twice with cold 10% TCA, twice with 95% ethanol, once with

absolute ethanol, and twice with anhydrous ethyl ether. The filters were then air dried, placed in a counting vial with 10 ml of Omniflour-toluene counting fluid and counted in a Packard model 3330 Tri-Carb Liquid Scintillation Spectrometer. ^{3}H-UTP and ^{14}C-ATP were purchased from either New England Nuclear Corp., Boston, Mass. or International Chemical and Nuclear Corp., Irvine, California. Unlabeled nucleoside triphosphates were purchased from Calbiochem, San Diego, California. Spermine tetrahydrochloride and putrescine dihydrochloride were purchased from Mann Research Laboratory, Inc., New York, New York. Cadaverine dihydrochloride and spermidine trihydrochloride was purchased from Calbiochem, San Diego, California.

Analysis of Complex-Bound Product RNA

Nitrocellulose filter analysis of complex-bound RNA was performed as described by Witmer (89) with some modifications. 10 µl aliquots were removed from the assay mixtures at the appropriate times and applied directly to B6 membrane filters (Schleicher and Schuell Inc., Keene, New Hampshire) which had been presoaked in 10 mM Tris-HCl, pH 7.9, 10 mM KCl. Washing with 50 ml of the same buffer immediately followed. Filters were dried by suction, followed by air drying and counting as previously described.

Sucrose Gradient Analysis of Reaction Mixture

2.75 µg of RNA polymerase was added to the complete reaction mixture (0.2 ml) containing 15 µg of ^{32}P-T4 DNA (1000 - 1500 dpm per µg) at staggered times and prepared for sucrose gradient analysis at the same time resulting in incubation periods of 5, 30 and 60 minutes. Two 10 µl aliquots were removed from each assay tube at the end of the incubation.

One 10 µl aliquot was used for analysis of complex bound RNA as previously described while the other 10 µl aliquot was used to determine total acid-precipitable counts. The remaining reaction mixtures were diluted to one ml with dilution buffer (50 mM Tris-HCl, pH 7.9, 40 mM KCl, and 1 mM DTT). 0.2 ml of this diluted reaction mixture was layered on a 5-20% sucrose gradient in the same buffer with a 0.2 ml 50% sucrose cushion and centrifuged for 105 minutes at 114,000 x G at 4° in a Spinco SW 50.1 rotor. Fractions were collected drop-wise from the bottom using a Hoefer Fractionator. (Hoefer Scientific, San Francisco, California). To each fraction 200 µg of bovine serum albumin (BSA) was added followed by 2.5 ml of cold 10% TCA. The precipitate was collected on Whatman GF/A glass fiber filters presoaked in 10% TCA and washed three times with 2.5 ml of 5% TCA. Filters were air dried and counted.

Sucrose Gradient Analysis of Product RNA Size

Sucrose gradient analysis of product RNA size was performed with two different gradient systems: NET-SDS gradients and formaldehyde gradients after Boedtker (97) as modified by Richardson (96). For analysis on NET-SDS [0.1 M NaCl, 0.1 mM EDTA, 0.01 M Tris-HCl, pH 7.9, and 0.5% (w/v) sodium dodecyl sulfate] gradients, 0.2 ml assay mixtures were incubated at 37° for 60 minutes at which time a 10 µl aliquot was removed for determination of total acid-precipitable counts. To the remainder of the reaction mixture 200 µg of 16 S and 23 S rRNA was added as sedimentation markers and the mixture was precipitated with 2 volumes of absolute ethanol and stored overnight at -20°. After centrifugation the resulting pellet was dissolved in 0.6 ml of NET-SDS buffer and incubated at 60° for 5 minutes to remove aggregates. A 0.3 ml aliquot was then layered on a 5-30% sucrose gradient in NET-SDS buffer and centrifuged

for 8.5 hours at 22° at 94,300 x G in a Spinco 27.1 rotor. 0.5 ml fractions were collected and 200 µg of BSA was added to each fraction followed by precipitation with 2.5 ml of cold 10% TCA and filtration through Whatman GF/A glass fiber filters. Filters were washed three times with 2.5 ml of cold 5% TCA, air dried, and counted in Omnifluor-toluene counting fluid. For analysis of RNA on formaldehyde gradients, 40 µl of 2.4% sodium dodecyl sulfate (SDS) was added at 60 minutes incubation time to the 0.2 ml assay mixtures after removal of 5 µl for determination of total acid-precipitable counts. After 3 minutes at 37° the samples were diluted to a final volume of 0.5 ml with 0.1 M sodium phosphate buffer, pH 7.7, and 3% formaldehyde (v/v) and heated to 65° for 15 minutes. The samples were then chilled and the SDS removed by centrifugation. 100 µl of the supernatant was layered on 5-20% sucrose gradients in the 0.1 M sodium phosphate, pH 7.7, 3% formaldehyde buffer and centrifuged for 5.5 hours at 189,000 x G in a Spinco SW 50.1 rotor at 4°. Fractions were collected and treated with BSA and cold TCA as previously described. 16 S and 23 S rRNA markers were centrifuged in parallel tubes.

DNA-RNA Hybridization

DNA-RNA hybridization studies were performed according to Gillespie and Spiegelman (114) with slight modifications. Product RNA used in hybridization studies was isolated from assay mixtures by a procedure suggested by Dr. D. D. Brown (personal communication). At the end of the incubation time the assay mixture remaining, (10 µl aliquots having been removed for total acid-precipitable count determination) was made 5 mM with $MgCl_2$ and RNase-free DNase was added to a concentration of 50 µg/ml. Following incubation at 30° for 15 minutes, this solution

was diluted with an equal volume of glass distilled water and made 0.5% with SDS. After chilling, an equal volume of H_2O saturated phenol was added with stirring at 4° for 15 minutes. This mixture was transferred to 12 ml tapered Corex centrifuge tubes and centrifuged for 20 minutes at 4,500 x G in an international 856 rotor at 4° to separate phases. The aqueous fraction was transferred to a thick walled 15 ml Corex centrifuged tube and the RNA was precipitated with 0.4 M NaCl and 2 volumes of cold absolute ethanol and stored overnight at -20°. The RNA was pelleted by centrifugation for 45 minutes at 4,500 x G and dissolved in 0.2 ml of 0.01 M Tris-HCl, pH 7.9. To remove any DNA that survived the DNase treatment the sample was placed in a boiling water bath for 2 minutes, quick cooled on ice and passed through a Millipore Swinny filter holder equipped with a 13 mm diameter type HA-0.45 μ Millipore filter (Millipore Corp., Bedford, Mass.). This RNA solution was now ready for use in hybridization studies.

DNA was denatured and immobilized on membrane filters as described by Gillespie and Spiegelman (114). Native DNA was denatured by incubation in solution A (2 ml of 6 N KOH per 100 ml of distilled water) at room temperature for 7 minutes. This denatured DNA solution was then neutralized with solution B (1 M Tris-HCl, pH 7.8, 20 x SSC, and 6 N HCl, 9:10:1) to give a final DNA concentration of 10 μg per ml (SSC = standard saline solution: 0.15 M NaCl, 0.015 M sodium citrate). 2 ml of this solution was applied to S-S type B-6 membrane filters presoaked in 4 x SSC, filtered slowly, washed with 10 ml of 4 x SSC, dried by suction, blotted on filter paper, and baked overnight at 70°. The amount of DNA immobilized on the membrane filter was analyzed by heating filters in 1.5 ml of 1 N HCl for 20 minutes at 100°, cooling and reading the

absorbance at 260 nm against a 1 N HCl blank. An absorbance value of 27 for a 1 mg per ml denatured DNA solution was used for the calculations.

Annealing was performed in counting vials by immersing filters in hybridization cocktail consisting of 0.5 ml of 2 x SSC, labeled product RNA, and distilled water to a final volume of 2 ml and incubating at 70° for 18 hours with constant shaking in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, Ill.). After incubation the filters were cooled and washed 5 times with 3 ml of 2 x SSC followed by a 30 minute incubation at room temperature with 50 µg/ml of ribonuclease-A (Sigma Chemical Co., St. Louis, Mo.) in 2 x SSC. The 2 x SSC wash and ribonuclease steps were repeated and following another 2 x SSC wash the filters were individually soaked in 10-15 ml of 2 x SSC at room temperature for 2 hours. The filters were then blotted dry, placed in counting vials, baked for 2 hours at 70° and counted as described previously.

Preparation of Template DNA

DNA from T4 phage was isolated using the method of Thomas and Abelson (115) with some modifications. For phage preparation, one liter of Hershey's medium (8 g Nutrient Broth, 5 g peptone, 1 g dextrose, 5 g NaCl, 0.4 g $MgCl_2$, 1 L distilled water) was inoculated with E. coli BB and grown at 37° to a cell density of 5 x 10^8 per ml (an absorbance of 0.09 at 620 nm with a Bausch and Lomb Spectronic 20). Tryptophan was added to a final concentration of 25 µg per ml and the cells were infected with T4 phage at a multiplicity of 0.1 followed by vigorous aeration at 37° until lysis was observed (3-4 hours). Complete lysis was effected by adding 3 ml of $CHCl_3$ per liter of medium and letting stand for one-half

hour. 0.3 ml of 1 M $CaCl_2$, 1 ml of 4 M $MgCl_2$ and 0.3 ml of a 1 mg per ml pancreatic DNase solution were then added per liter of media followed by incubation for 30 minutes at 37°. Centrifugation at 5,117 x G in a Sorval type GS-3 rotor for 20 minutes at 4° followed incubation. The supernatant was centrifuged at 13,701 x G for 2 hours to pellet the phage. The resulting supernatant was carefully removed and the tube walls rinsed with a buffer consisting of 10 mM Tris-HCl, pH 7.9, 10 mM KCl, and 5 mM $MgCl_2$. This buffer was added sufficiently to cover the pellet and the phage were resuspended by shaking very slowly overnight at room temperature on a Gyrotory Model S-3 shaker (New Brunswick Scientific Co., New Brunswick, N.J.). The phage suspension was centrifuged at 5,117 x G in a Sorval SS-1 rotor for 20 minutes and the resulting supernatant was centrifuged at 27,000 x G in the same rotor for one hour. The phage pellet was resuspended as before and this concentrated stock suspension was stored at 4° over a drop of $CHCl_3$ to maintain sterility. For preparation of ^{32}P-T4 DNA, phage were prepared as before except that 3 mCi of ^{32}P-phosphate (New England Nuclear, Boston, Mass.) was added per liter of medium at the time of infection.

DNA was extracted from the phage suspension (1-2 x 10^{12} phage per ml) by added an equal volume of phenol saturated with 100 mM Tris-HCl, pH 7.9, 100 mM NaCl, and 2.0 mM EDTA followed by gentle shaking on the Dubnoff shaking incubator at room temperature for 30 minutes. This solution was cooled to 0° and centrifuged for 5 minutes at 1,085 x G to separate phases and the bottom phenol layer was removed. Extraction was repeated twice more for 15 and 8 minutes. Following the removal of the phenol layer of the third extraction, the aqueous layer was centrifuged for 40 minutes at 27,000 x G in a Sorval SS-1 rotor at 4°. The top 75%

of the DNA solution was then transferred to dialysis tubing (previously boiled for 20 minutes in 5% $NaHCO_3$ and thoroughly washed) and dialyzed exhaustively against 5 mM Tris-HCl, pH 7.9. The dialyzed DNA was then stored at 4° over one drop of $CHCl_3$. Absorption spectra were obtained on all DNA preparations and in all cases the 260/280 ratios exceeded 1.75. To minimize shearing of intact DNA molecules, the solutions were transferred by pouring rather than by pipetting.

T7 phage were prepared by infecting one liter of E. coli BB, at a cell density of 5×10^8/ml (with a multiplicity of 0.1) after addition of tryptophan to a concentration of 25 μg per ml. Lysis was rapid, usually within 1-1.5 hours. Following lysis, 3 ml of $CHCl_3$ was added per liter of medium. After standing for 30 minutes at 37° the medium was centrifuged at 5,117 x G for 20 minutes. The supernatant was brought to 0.5 M with NaCl and 10% with polyethylene glycol 6000 (J. T. Baker Chemical Co., Phillipsburg, N.J.) and chilled to 4° overnight. The polyethylene glycol mixture was centrifuged at 5,117 x G for 40 minutes and the pellet resuspended in at least 20 volumes of Nomura salts. The phage suspension was then centrifuged at 5,117 x G in a Sorval SS-1 rotor for 20 minutes and the resulting supernatant centrifuged for 3 hours at 39,100 x G to pellet the phage. The pellet was resuspended, centrifuged again at low speed, and the supernatant was layered on a CsCl gradient (density range 1.38 - 1.70 g per cc) and centrifuged for 75 minutes at 87,651 x G in a Spinco SW 65 T rotor. The phage band was collected, diluted to an absorbance of 25 at 260 nm, and dialyzed against a buffer containing 10 mM Tris-HCl, pH 7.9, 10 mM KCl and 5 mM $MgCl_2$. The purified T7 phage DNA was extracted using the same procedure as for T4 DNA. Each T4 and T7 phage preparation were started from a single plaque.

E. coli BB, T4 phage, and T7 phage were supplied by Dr. D. MacDonald Green and all phage preparations were conducted in his laboratory.

Purchased nucleic acids used in these studies included highly polymerized calf thymus DNA, *E. coli* strain B tRNA-stripped (General Biochemical, Chagrin Falls, Ohio), *E. coli* DNA, and Poly [d(A/T)] (P. L. Biochemical, Inc., Milwaukee, Wis.).

Highly purified *E. coli* 16 S and 23 S ribosomal RNA was a generous gift to Dr. Herbst from Dr. Volker Erdmann.

RESULTS

Section I: Influence of Cations on *in vitro* Transcription

It is well documented (23, 78, 82, 89, 94, 100) that *in vitro* transcription by *E. coli* DNA-dependent RNA polymerase on native DNA templates exhibits early plateauing at low ionic strength conditions. (See Methods section for definition of low and high ionic strength conditions). That is, RNA synthesis appears to be linear for the first 10 to 15 minutes of the reaction followed by a rapid leveling off and near cessation of synthesis by 60 minutes.

Examples of early plateauing and prevention of plateauing by the addition of the organic triamine spermidine [H_2N-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2] or the monovalent salt KCl to the reaction mixture are illustrated in Figure 1. The presence of 0.3 M KCl in the reaction mixture results in a continuation of RNA synthesis in a near linear fashion for 120 minutes with a slight reduction in the rate of synthesis observed from 120 minutes to 240 minutes. Other investigators have reported this type of kinetic behavior at elevated ionic strength with KCl, NH_4Cl, and $MgCl_2$ (23, 78, 82, 89, 100, 101). 2.5 mM spermidine is also effective in preventing the early appearance of a plateau, but the stimulation of RNA synthesis is less pronounced.

Figures 2 and 3 show the response elicited by increasing concentrations of KCl and spermidine respectively on the transcription of T4 DNA. At both 60 and 120 minutes essentially no stimulation of RNA synthesis is observed at concentrations of 0.1 M KCl or below. The presence of KCl

FIGURE 1

Time course of RNA synthesis showing early plateauing and prevention of plateauing by 2.5 mM spermidine and 0.3 M KCl: the reaction mixtures contained 50 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$; 1 mM ATP, GTP, and CTP; 0.5 mM ^{3}H-UTP (1.8×10^6 dpm); 0.1 mM dithiothreitol (DTT); 15 µg T4 DNA; 2.5 µg RNA polymerase; and spermidine and KCl as indicated. Incubation was at 37° for four hours with removal of 10 µl aliquots at time points indicated. Aliquots were prepared for counting as described in the Methods section. (○) low salt control (0 KCl), (□) low salt + 2.5 mM spermidine, (▽) high salt (0.3 M KCl).

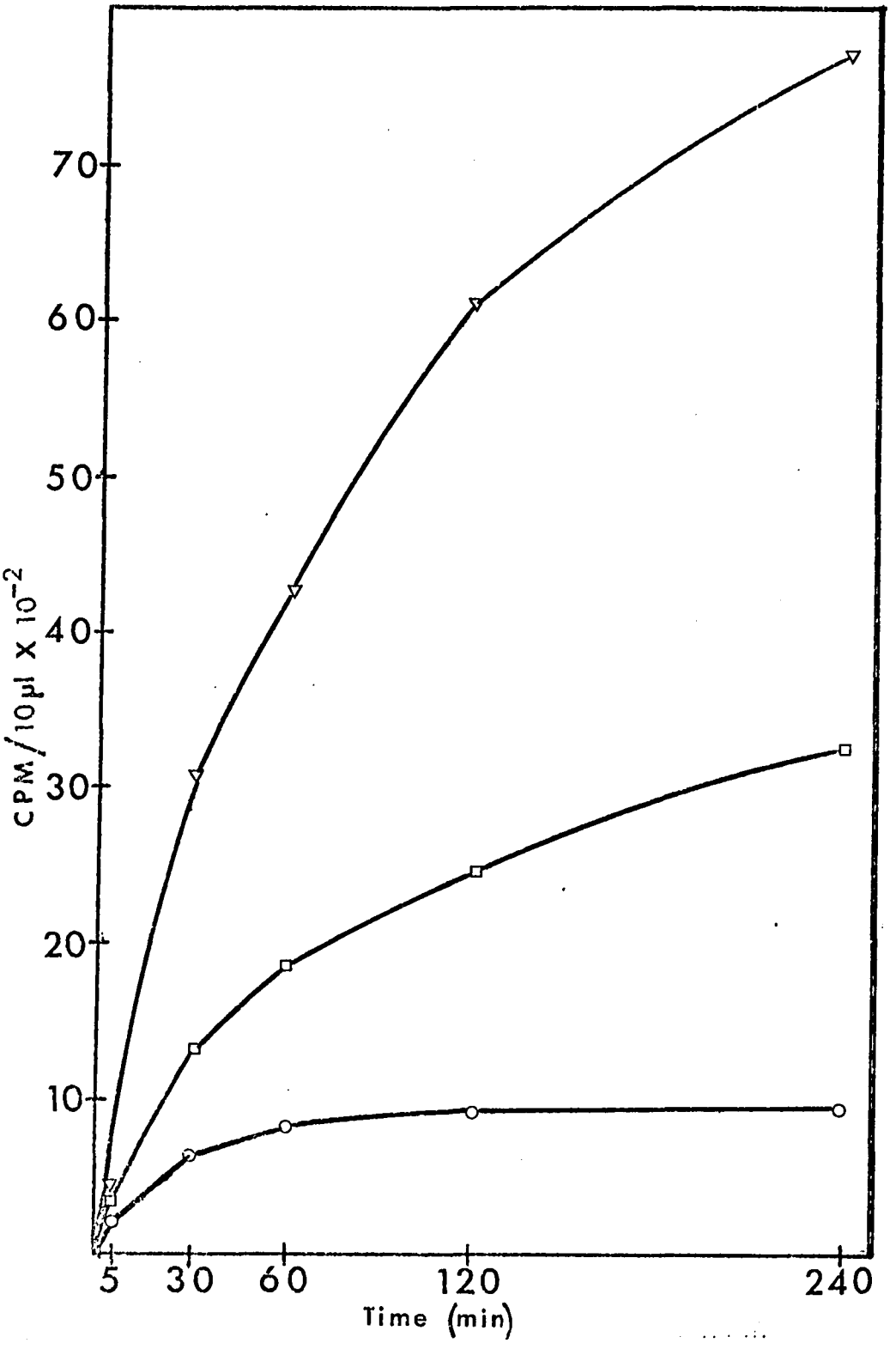

70
60
50
40
30
20
10
CPM/10µl X 10⁻²
5
30
60
120
240
Time (min)

FIGURE 2

KCl dose response curve. Reaction mixture and assay conditions were as described for Figure 1. (O) 60 minutes incubation, (●) 120 minutes incubation time.

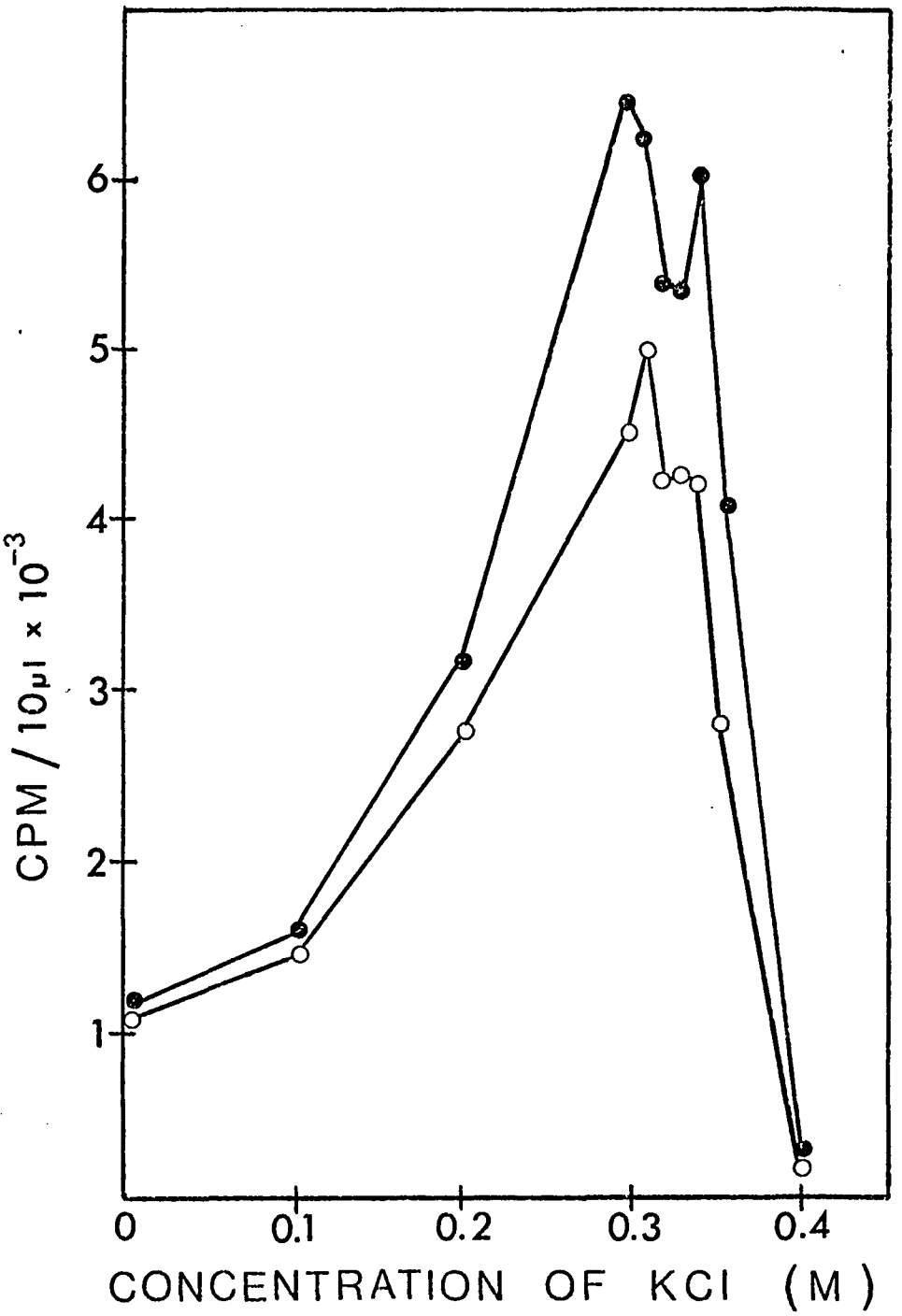
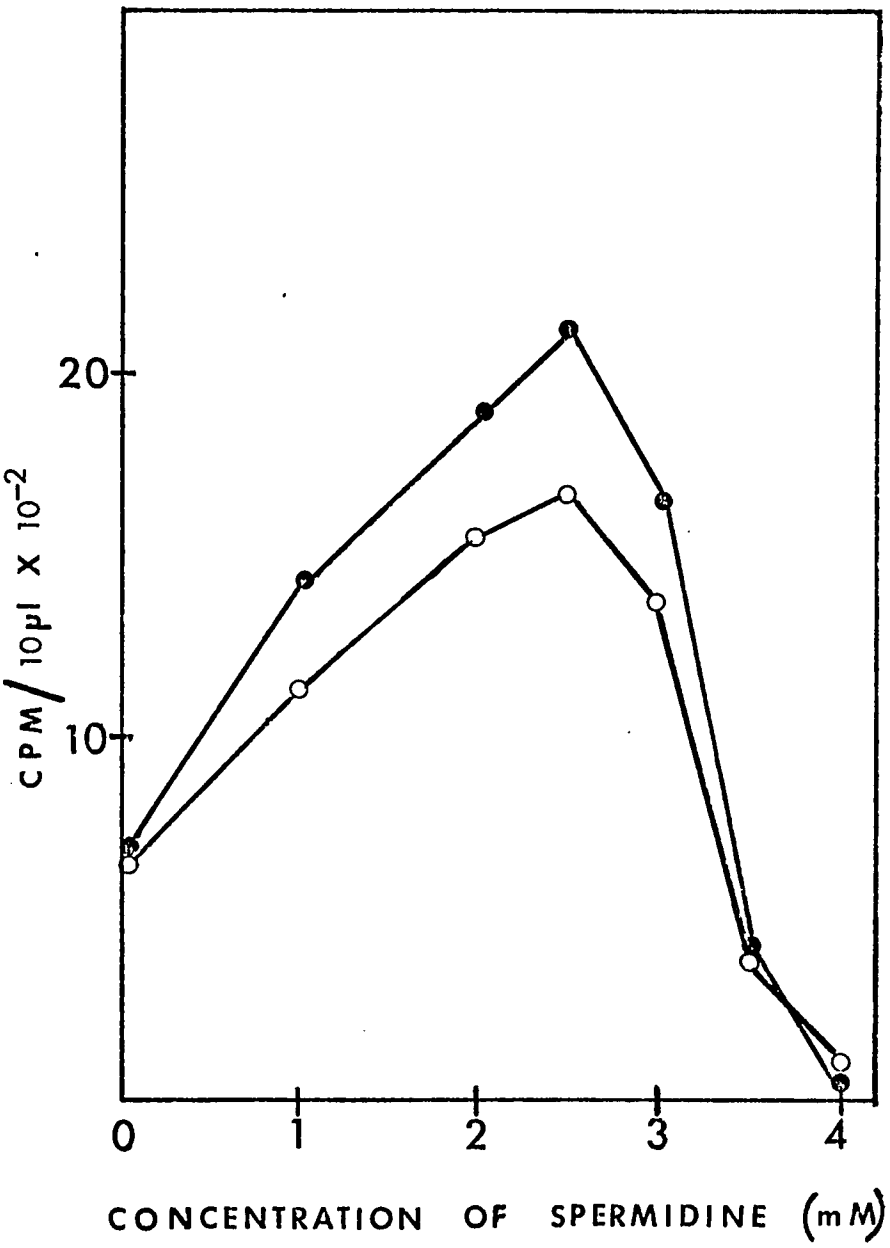

CPM / 10µl × 10⁻³
6
5
4
3
2
1
0
0.1
0.2
0.3
0.4
CONCENTRATION OF KCl (M)

FIGURE 3

Spermidine dose response curve. Reaction mixture and assay conditions were as described for Figure 1. (O) 60 minutes incubation time, (●) 120 minutes incubation time.


20
10
CPM / 10µl X 10^{-2}
0
1
2
3
4
CONCENTRATION OF SPERMIDINE (mM)

at concentrations above 0.1 M results in stimulation of RNA synthesis and optimum rates are observed at 0.3 M-0.31 M KCl. When spermidine is added to the low ionic strength reaction mixture (Figure 3), a stimulatory response is observed at concentrations ranging from 1 mM to 3 mM with optimum stimulation observed at a concentration of 2.5 mM. At concentrations above 3 mM a precipitate, presumably of the T4 template, is visible resulting in a marked reduction in synthetic activity.

Because spermidine stimulates in vitro transcription in a much less pronounced fashion than KCl, at least one investigator has dismissed the spermidine-mediated stimulation as a minimal "salt" effect (17). This interpretation seems inadequate in view of the 100-fold difference in the effective concentration of KCl and spermidine.

A major difficulty in the establishment of optimum spermidine concentrations can be traced to the problem of template precipitation. Table I shows that when an inhibitory concentration (6 mM) of spermidine is added to a reaction mixture containing various concentrations of KCl, synthesis at each KCl concentration is markedly enhanced. Interestingly enough, at a concentration of 0.1 M KCl, a concentration at which early cessation of synthesis is observed, the addition of 6 mM spermidine results in a stimulation of RNA synthesis in excess of that observed at an apparent optimal KCl concentration of 0.3 M. Furthermore, additional stimulation by spermidine is observed in assays containing 0.3 M KCl.

It can be concluded that the elevation of ionic strength "protects" the template present in the reaction mixture from precipitation by spermidine. Moderately increased concentrations of KCl, ineffective in stimulating RNA synthesis allow spermidine-mediated stimulation of RNA synthesis comparable to that observed at high concentrations of KCl. It

TABLE I

Spermidine Enhancement of KCl - Stimulated Transcription*

KCl (M)	Spermidine (mM)	Incubation Time (min)			
		30	60	120	240
		cpm (+ cations) + cpm (- cations) x 100			
0	6	23	29	28	21
0.1	0	103	115	100	103
0.2	0	156	177	299	282
0.3	0	332	388	530	555
0.1	6	272	413	498	583
0.2	6	366	356	547	619
0.3	6	325	555	892	916

* The reaction mixtures and assay conditions were as described for Figure 1.

is significant that millimolar concentrations of spermidine can effect such large stimulatory responses at moderate and optimal KCl concentrations.

At this point, it is of interest to investigate the properties of cogeners of spermidine such as the diamines putrescine [H_2N-$(CH_2)_4$-NH_2] and cadaverine [H_2N-$(CH_2)_5$-NH_2] and the tetramine spermine [H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2]. Data comparing the effect of these amines and spermidine at four incubation times at both high and low salt conditions are presented in Tables II through IX. A large number of amine concentrations at several incubation times are compared in order to provide a general overview of the relative activity of these amines. A summary of these data at one incubation time is presented graphically

in Figures 4 and 5.

Putrescine, as shown in Table II, effectively stimulates RNA synthesis over a concentration range of 60 to 90 mM. By 240 minutes of incubation 80 mM putrescine is able to stimulate [^{3}H]-UMP incorporation by a factor of 10. Putrescine is also stimulatory at high ionic strength (Table III), with a maximum stimulation of 2.95 fold observed at a concentration of 30 mM. Above a concentration of 30 mM, putrescine is found to cause inhibition of synthesis in reactions containing 0.3 M KCl. However, it must be pointed out that the amount of RNA synthesized at high salt-putrescine conditions is still substantially greater than that obtained at low salt conditions.

TABLE II

Stimulation of RNA Synthesis by Putrescine: Low Salt Conditions (0 KCl)*

Concentration of Putrescine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ putrescine) ÷ cpm (- putrescine) x 100			
10	115	137	150	172
20	157	185	213	194
30	195	249	276	271
50	217	392	406	---
60	346	417	549	660
70	393	496	566	731
80	424	569	822	1,084
90	500	561	808	1,077
100	169	210	316	387

* The reaction mixture and assay conditions were as described in Figure 1.

TABLE III

Stimulation of RNA Synthesis by Putrescine: High Salt Conditions (0.3 M KCl)*

Concentration of Putrescine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ putrescine) ÷ cpm (- putrescine) x 100			
5	117	113	103	110
10	127	127	135	154
15	131	141	145	161
20	118	138	154	167
30	171	199	237	295
40	28	39	41	47
50	16	18	20	23

* The reaction mixture and assay conditions were as described for Figure 1 with the addition of 0.3 M KCl.

Tables IV and V show that cadaverine, although not as effective as putrescine, does exhibit optimum stimulatory concentrations in approximately the same range as putrescine at both high and low salt conditions. At low salt conditions, a maximum stimulation of 7.09 over the control value is observed at 240 minutes by 100 mM cadaverine while, at high salt conditions, concentrations between 10 and 20 mM are optimum.

Spermidine, at a concentration of 2.5 mM results in a stimulation of 3.66 over the low salt control value (Table VI) and while concentrations as low as 1 mM are stimulatory, concentrations above 3 mM inhibit RNA synthesis. As previously mentioned, this inhibition is associated with a clearly visible precipitate observed in assay mixtures containing these concentrations of spermidine. At high salt conditions (Table VII), however, no precipitation is observed and spermidine exhibits a stimulatory

TABLE IV

Stimulation of RNA Synthesis by Cadaverine: Low Salt Conditions (0 KCl)*

Concentration of Cadaverine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ cadaverine) ÷ cpm (- cadaverine) x 100			
10	117	124	131	91
20	171	183	200	186
30	173	207	209	212
50	188	225	220	239
60	240	320	293	319
70	276	343	367	424
90	317	362	422	455
100	304	429	560	709
150	27	25	37	40

* The reaction mixture and assay conditions were as described for Figure 1.

TABLE V

Stimulation of RNA Synthesis by Cadaverine: High Salt Conditions (0.3 M KCl)*

Concentration of Cadaverine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ cadaverine) ÷ cpm (- cadaverine) x 100			
10	128	137	149	155
20	78	90	105	121
25	46	51	56	66
30	38	48	53	56
40	16	19	24	25

* The reaction mixture and assay conditions were as described for Table III.

TABLE VI

Stimulation of RNA Synthesis by Spermidine: Low Salt Conditions (0 KCl)*

Concentration of Spermidine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ spermidine) ÷ cpm (- spermidine) x 100			
0.5	101	106	---	---
1.0	145	176	203	244
2.0	197	243	275	329
2.5	212	262	308	366
3.0	162	228	233	269
3.5	40	63	69	88
4.0	26	15	13	12

* The reaction mixture and assay conditions were as described for Figure 1.

TABLE VII

Stimulation of RNA Synthesis by Spermidine: High Salt Condition (0.3 M KCl)*

Concentration of Spermidine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ spermidine) ÷ cpm (- spermidine) x 100			
2	105	127	135	127
3	135	129	141	152
4	142	145	167	163
6	140	144	196	225
8	118	138	178	196
10	119	118	143	155

* The reaction mixture and assay conditions were as described for Table III.

response over a large range of concentrations showing a stimulation of 1.55 over the high salt control value even at 10 mM, and a 2.5 fold stimulation at an optimum concentration of 6 mM.

Spermine at low ionic strength conditions, is 10 times more effective than spermidine in stimulating [^{3}H]-UMP incorporation exhibiting a maximum 3.29 fold stimulation of the control reaction at a concentration of 0.25 mM (Table VIII). Above this concentration spermine is very inhibitory also causing the appearance of a visible precipitate in the reaction mixture. At high ionic strength conditions (Table IX) spermine behaves similarly to spermidine exhibiting a stimulatory response of 2.79 over the high salt control value at a concentration of 6 mM.

TABLE VIII

Stimulation of RNA Synthesis by Spermine: Low Salt Conditions (0 KCl)*

Concentration of Spermine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ spermine) ÷ cpm (- spermine) x 100			
0.10	131	155	169	186
0.25	184	232	275	329
0.50	13	13	13	25
1.00	10	9	11	14
2.00	9	8	8	32
3.00	10	7	14	17

* The reaction mixture and assay conditions were as described for Figure 1.

TABLE IX

Stimulation of RNA Synthesis by Spermine: High Salt Concentration (0.3 M KCl)*

Concentration of Spermine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ spermine) ÷ cpm (- spermine) x 100			
0.10	101	95	101	91
0.25	105	93	106	96
0.50	126	120	136	137
1.00	142	152	179	174
2.00	152	156	194	219
3.00	139	169	206	273
4.00	110	152	199	242
6.00	140	227	232	279

* The reaction mixture and assay conditions were as described for Table III.

The inorganic divalent cation Mg^{++} has also been reported to stimulate *in vitro* RNA synthesis (77, 78). Furthermore, Mg^{++} and spermidine appear to have similar roles in diverse interactions of nucleic acids (93, 117, 120). It was of interest, therefore, to compare the activity of Mg^{++} and spermidine in the *in vitro* transcription systems.

Tables X and XI show that Mg^{++} is similar, with respect to the level of stimulation and concentration range, to the organic divalent cations, putrescine and cadaverine, at both salt conditions. At low KCl concentrations, Mg^{++} is stimulatory over a concentration range of 50 to 100 mM with an optimum stimulation of 12.64 over the control observed at a concentration of 80 mM. A concentration range of 10 to 20 mM appears to be optimal at high KCl concentrations while a concentration of 50 mM

TABLE X

Stimulation of RNA Synthesis by $MgCl_2$: Low Salt Conditions (0 KCl)*

Concentration of $MgCl_2$ (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ $MgCl_2$) ÷ cpm (- $MgCl_2$) x 100			
10	111	131	143	145
15	123	182	---	---
20	152	186	205	230
50	268	401	551	649
60	252	372	485	686
70	216	420	599	803
80	178	573	868	1,264
90	61	383	629	507
100	48	220	282	370

* The reaction mixture and assay conditions were as described for Figure 1 for the control tube. In the remaining reaction vessels, 5 mM $MgCl_2$ is replaced by the $MgCl_2$ concentrations indicated.

TABLE XI

Stimulation of RNA Synthesis by $MgCl_2$: High Salt Conditions (0.3 M KCl)*

Concentration of $MgCl_2$ (mM)	Incubation Time (min)			
	30	60	120	240
	cpm (+ $MgCl_2$) ÷ cpm ($MgCl_2$) x 100			
10	127	133	153	161
15	123	163	178	185
20	127	170	164	185
30	41	44	50	50
40	17	22	23	21
50	7	8	12	8

* The reaction mixture and assay conditions were as described for Table III for the control tube. In the remaining reaction tubes 5 mM $MgCl_2$ is replaced by the $MgCl_2$ concentrations indicated.

inhibits RNA synthesis to a level even below that of the low salt control.

The responses elicited by these compounds with regard to concentration range and level of stimulation of the in vitro RNA synthetic reaction can be arranged into several classes or groups. One class is represented by KCl which is active in preventing early cessation of synthesis at concentrations above 0.1 M and stimulating RNA synthesis some 7-8 fold at a concentration of 0.3 M. Other investigators have reported similar results for NH_4Cl, (78).

A second class is represented by the divalent cations Mg^{++}, putrescine, and cadaverine which exhibit a 6 to 12 fold stimulation of RNA synthesis at concentrations ranging from 50 to 100 mM. These compounds are also stimulatory in the presence of 0.3 M KCl giving incorporation values 1.5 to 2 fold greater than the high salt control value at concentrations ranging from 10 to 30 mM.

A third category is represented by the polyamines spermine and spermidine which exhibit stimulation of 2 to 3 fold at concentrations of 0.25 mM and 2.5 mM respectively. At concentrations above the apparent optimum, a visible precipitate is observed in the reaction mixture resulting in inhibition of RNA synthesis. Higher concentrations of spermidine and, presumably spermine, in the presence of low concentrations (0.05 - 0.1 M) of KCl, which prevent the appearance of any precipitate in the reaction mixture, stimulate RNA synthesis by 8 to 10 fold. Both amines also appear to stimulate RNA synthesis 2 to 3 fold even at an elevated ionic strength (0.3 M KCl).

Figures 4 and 5 present in graphic form the stimulatory effect of class 2 and 3 compounds respectively at one incubation time (120 min.) and at both KCl concentrations. The dose response profiles of Mg^{++} and

FIGURE 4

Dose response curves for $MgCl_2$, putrescine, and cadaverine at 120 minutes at both low [A] and high [B] salt conditions. Reaction mixtures and assay conditions were as described for Tables II-V and X-XI. (□) and (■) $MgCl_2$, (O) and (●) putrescine, (▽) and (▼) cadaverine.

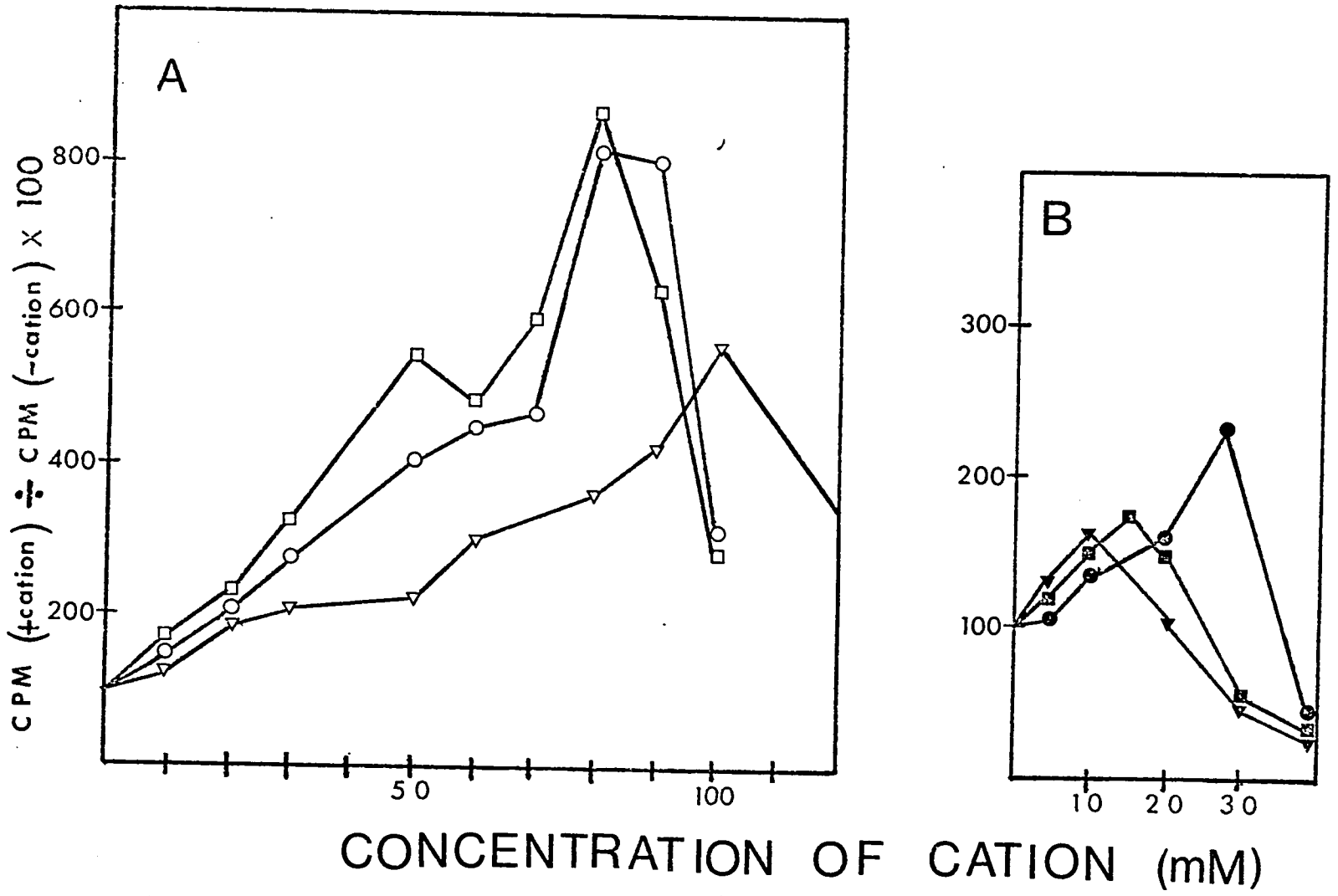
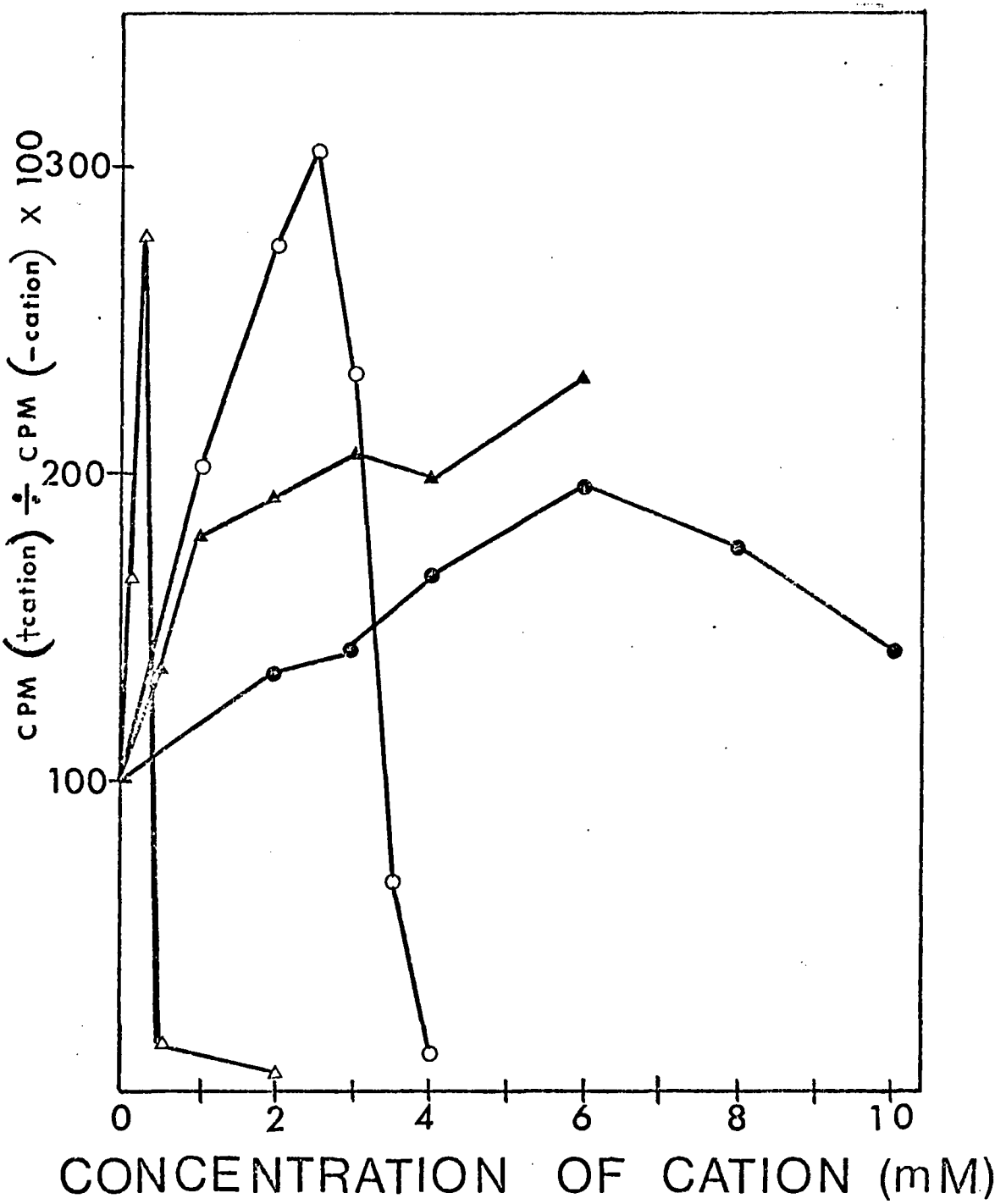

A
B
CPM (+cation) ÷ CPM (-cation) X 100
800
600
400
200
50
100
300
200
100
10
20
30
CONCENTRATION OF CATION (mM)

FIGURE 5

Dose response curves for spermine and spermidine at 120 minutes at both low and high salt conditions. Reaction mixtures and assay conditions were as described for Tablex VI - IX. (Δ) spermine low salt conditions, (▲) spermine high salt conditions, (O) spermidine low salt conditions, (●) spermidine high salt conditions.


CPM (+cation) ÷ CPM (-cation) X 100
300
200
100
0
2
4
6
8
10
CONCENTRATION OF CATION (mM)

putrescine (Figure 4) are strikingly similar at both KCl concentrations. Cadaverine requires slightly higher concentrations for maximum stimulations but in all three cases the optimum concentration is much lower in the presence of 0.3 M KCl than in its absence. Spermidine and especially spermine (Figure 5) show sharp optima at low ionic strength and very broad dose response profiles at high KCl concentrations.

Since class 2 and class 3 compounds were stimulatory, even at high KCl concentrations, it was of interest to see whether a member of class 3, e.g., spermidine, would be stimulatory in the presence of optimum concentrations of a class 2 compound, e.g., Mg^{++}, at either high or low KCl concentrations. Table XII indicates that spermidine is not stimulatory at an optimum Mg^{++} concentration of 80 mM in the absence of KCl. In fact, the level of RNA synthesis tends toward low salt control values as the spermidine concentration is increased. Similar results are evident at high KCl concentrations (Table XIII) in the presence of 20 mM $MgCl_2$. However, it should be pointed out that in experiments with added spermidine in the presence of suboptimal concentrations of $MgCl_2$, RNA synthesis is observed to reach levels higher than that observed at optimum concentrations of either compound alone.

At least one investigator (116) has dismissed the possibility that polyamines act in a rather specific manner to stimulate <u>in vitro</u> RNA synthesis by attributing the action of polyamines simply to their contribution to the net ionic strength. Figure 6 conveniently shows that a strict relationship between ionic strength and <u>in vitro</u> RNA synthetic activity does not exist. In all cases, the presence of polyamines or divalent cations results in a much greater contribution to the synthetic activity than to the net ionic strength of the reaction media.

TABLE XII

Stimulation of RNA Synthesis at Optimum $MgCl_2$ Concentration (80 mM) by Spermidine: Low Salt Conditions (0 KCl)*

Concentration of Spermidine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm/10λ			
0	1,143	3,292	5,805	8,116
2	1,081	2,690	5,090	7,395
3	1,624	3,004	4,415	6,040
5	896	2,444	4,690	6,430
6	855	2,480	4,049	6,053
8	719	1,755	2,760	3,756
10	598	1,426	1,941	2,366

* The reaction mixtures contained 50 mM Tris-HCl (pH 7.9); 80 mM $MgCl_2$; 1 mM ATP, CTP, and GTP; 0.5 mM ^{3}H-UPT (1.8×10^6 dpm); 0.1 mM DTT; 15 µg T4 DNA; 2.5 µg of RNA polymerase; and the indicated concentration of spermidine.

TABLE XIII

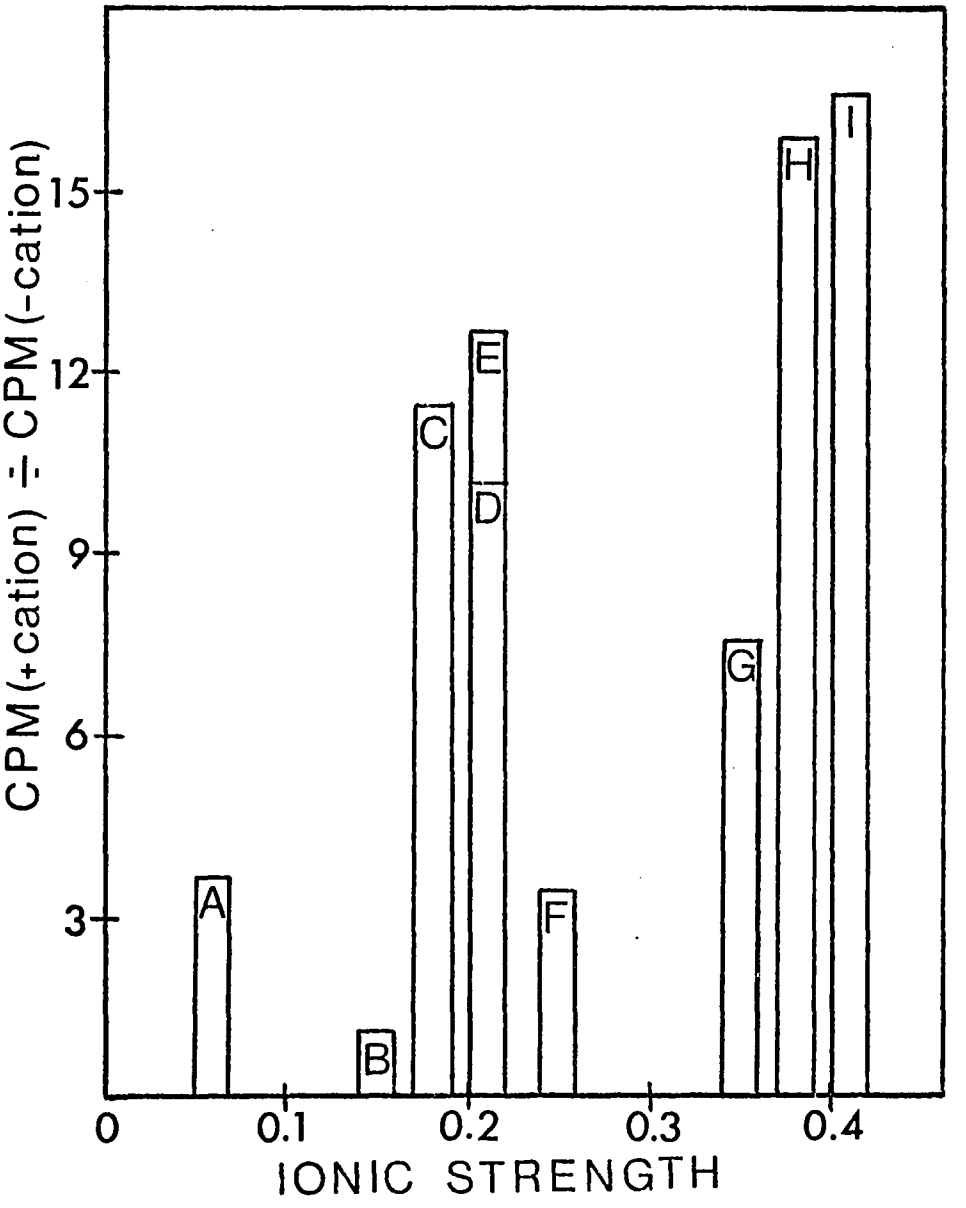
Stimulation of RNA Synthesis at Optimum $MgCl_2$ Concentration (20 mM) by Spermidine: High Salt Condition (0.3 M KCl)*

Concentration of Spermidine (mM)	Incubation Time (min)			
	30	60	120	240
	cpm/10λ			
0	2,727	5,309	6,925	9,643
2	2,235	4,247	6,412	9,094
3	2,668	4,843	6,275	8,277
5	2,088	3,918	5,621	8,276
6	1,831	3,193	3,989	5,483
8	1,401	2,366	3,147	4,039
10	291	523	601	639

* Reaction mixtures contained 50 mM Tris-HCl (pH 7.9); 20 mM $MgCl_2$; 1 mM ATP, CTP, and GTP; 0.5 mM ^{3}H-UTP (1.8×10^6 dpm); 0.1 mM DTT; 0.3 M KCl; 15 µg T4 DNA; 2.5 µg RNA polymerase; and the indicated concentration of spermidine.

FIGURE 6

Relationship of ionic strength and RNA polymerase synthetic activity: the various reaction mixture contain: (A) 2.5 mM spermidine, (B) 0.1 M KCl, (C) 0.1 M KCl + 6 mM spermidine, (D) 80 mM putrescine, (E) 80 mM $MgCl_2$, (F) 0.2 M KCl, (G) 0.3 M KCl, (H) 0.3 M KCl + 6 mM spermidine, and (I) 0.3 M KCl + 30 mM putrescine.


CPM(+cation) ÷ CPM(-cation)
15
12
9
6
3
A
B
C
D
E
F
G
H
I
0
0.1
0.2
0.3
0.4
IONIC STRENGTH

Section II: Comparison of KCl and spermidine-mediated stimulation of *in vitro* RNA synthesis.

Data previously presented in this thesis (Figure 6) strongly indicates that the activity of polyamines in stimulating *in vitro* RNA synthesis cannot simply be attributed to their contribution to the net ionic strength. However, it was still felt that any experiments conducted in an effort to determine the mechanism of action of polyamine-mediated stimulation should be designed so as to allow direct comparison with the effect of KCl.

Figure 7 summarizes the results from a large number of experiments performed with several preparations of T4 DNA template and *E. coli* RNA polymerase. Stimulation by 2.5 mM spermidine appears to be nearly linear over most of the time course, with little effect observed during the first few minutes of incubation. Stimulation by 0.3 M KCl, on the other hand, occurs primarily in the first 60 minutes of the four hour incubation period. The stimulatory response to 6 mM spermidine in the presence of 0.1 M KCl appears to be very similar to that obtained with 0.3 M KCl in the first hour of synthesis; however, more activity is observed after 60 minutes in the former case. 6 mM spermidine in the presence of 0.3 M KCl shows comparatively little stimulation in the first 20 minutes, but, by 60 minutes more, activity is observed than is exhibited by 0.3 M KCl, and considerable activity is observed in the ensuing 3 hours.

Figure 8 shows the initial rate of RNA synthesis as influenced by 0.1 M KCl, 0.1 M KCl + 6 mM spermidine and 0.3 M KCl. The incubation temperature in these experiments is 25° rather than 37°, reducing the reaction rate for a more critical examination of early kinetics. Synthesis

FIGURE 7

Summary of kinetic behavior exhibited at various spermidine and KCl conditions. The reaction mixture and assay conditions have previously been described. Data is presented as cpm incorporated in presence of KCl, spermidine, or both divided by cpm incorporated in their absence all multiplied by 100. (O) 2.5 mM spermidine, (□) 0.3 M KCl, (●) 6 mM spermidine + 0.1 M KCl, (▲) 6 mM spermidine + 0.3 M KCl. The calculated ionic strength for each reaction mixture is: 0.05 for the low salt control, 0.06 for the 2.5 mM spermidine assay, 0.18 for the assay containing 0.1 M KCl + 6 mM spermidine, 0.35 for the 0.3 M KCl reaction, and 0.38 for the assay containing 0.3 M KCl + 6 mM spermidine.

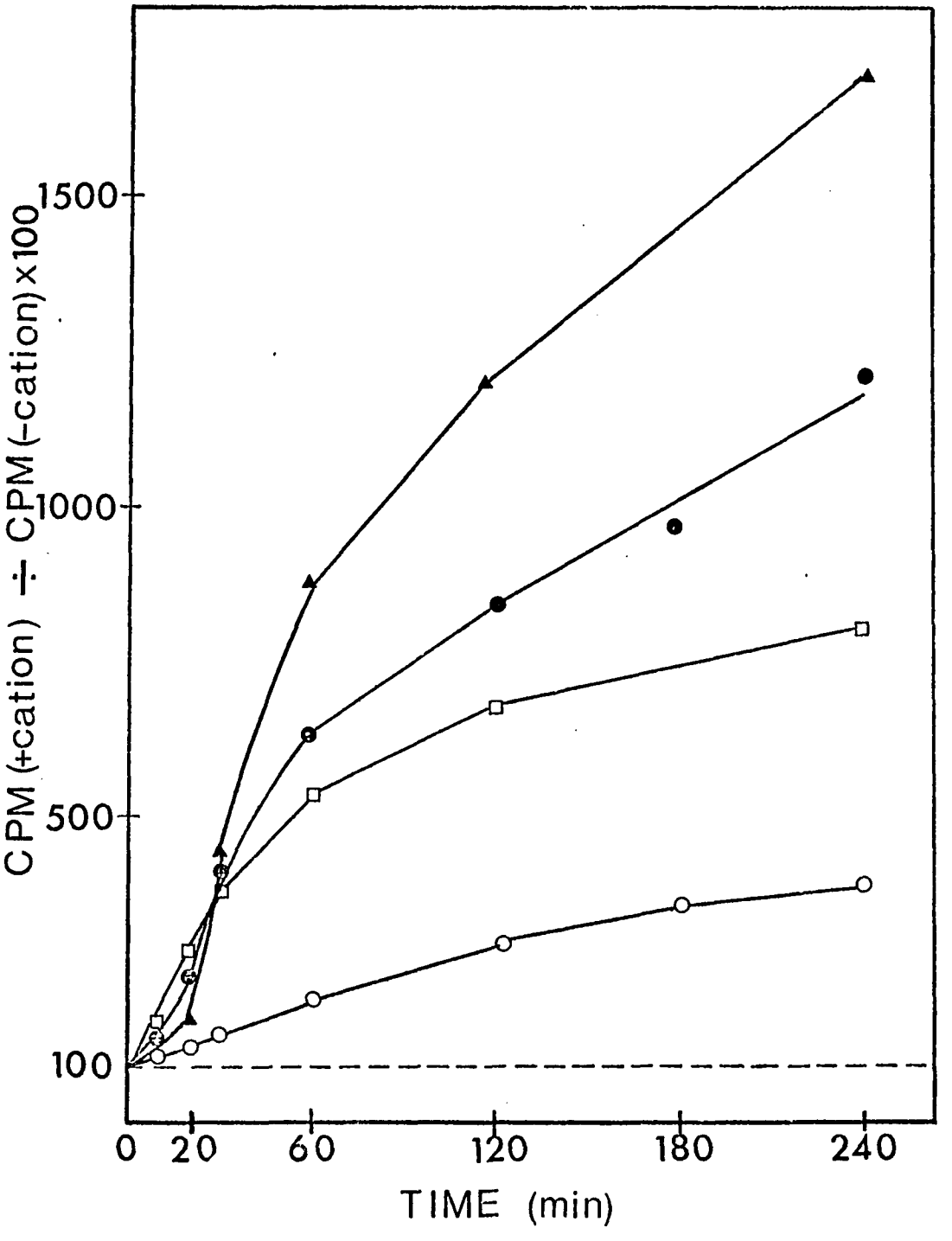
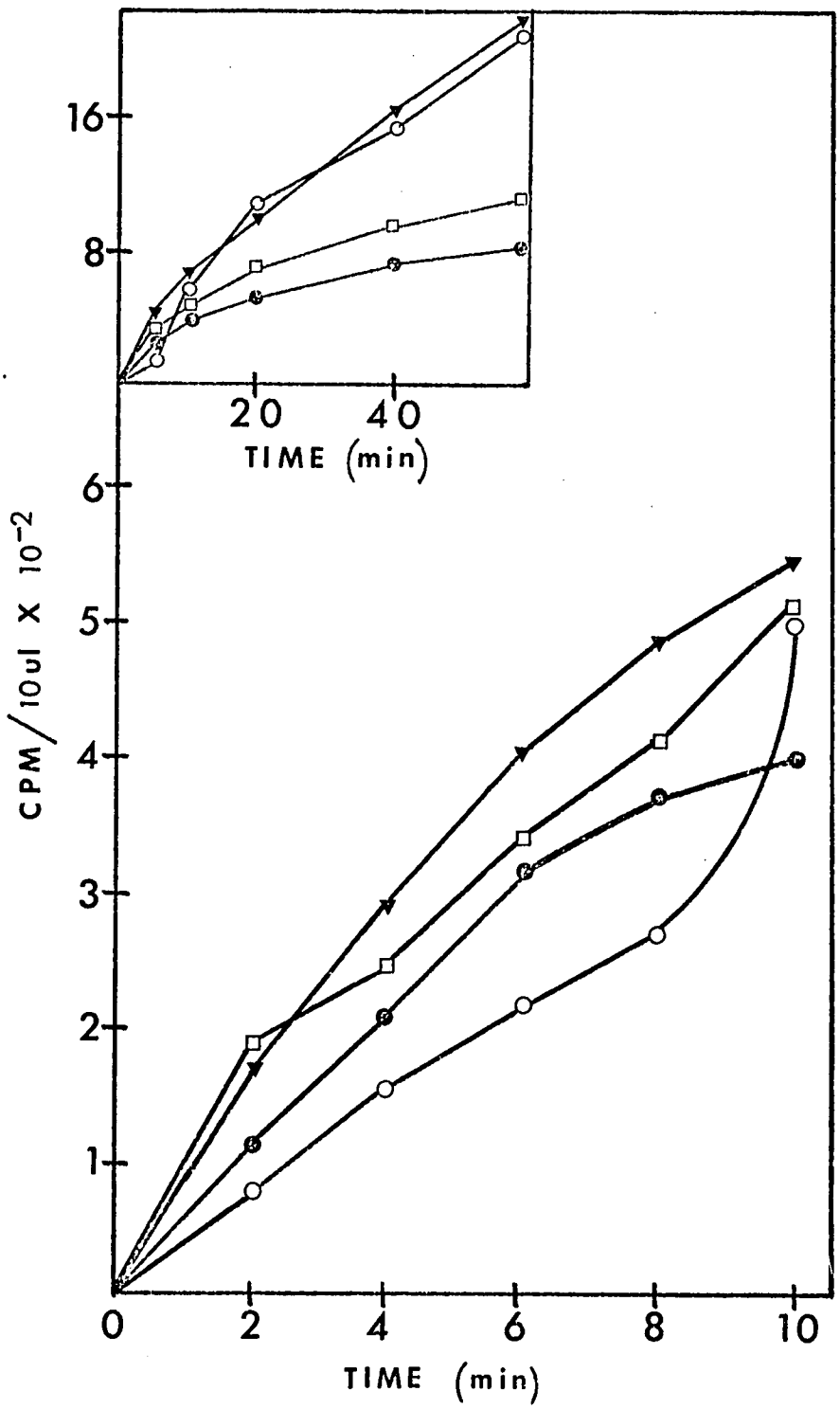

CPM (+cation) ÷ CPM (-cation) x100
1500
1000
500
100
0
20
60
120
180
240
TIME (min)

FIGURE 8

Comparison of initial rates exhibited by reaction mixtures containing 0 KCl (●), 0.1 M KCl (□), 0.3 M KCl (○), or 6 mM spermidine + 0.1 M KCl (▼). The reaction mixtures contained 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 0.2 mM GTP, CTP, and UTP; 0.2 mM ^{14}C-ATP (1.2×10^5 dpm); 0.1 mM DTT; 14 µg T4 DNA; 5 µg RNA polymerase; and the indicated amounts of spermidine and KCl. The incubation temperature was 25°. Inset shows the extended time course of same experiment.


16
8
20
40
TIME (min)
6
5
4
3
2
1
CPM/10ul X 10^{-2}
0
2
4
6
8
10
TIME (min)

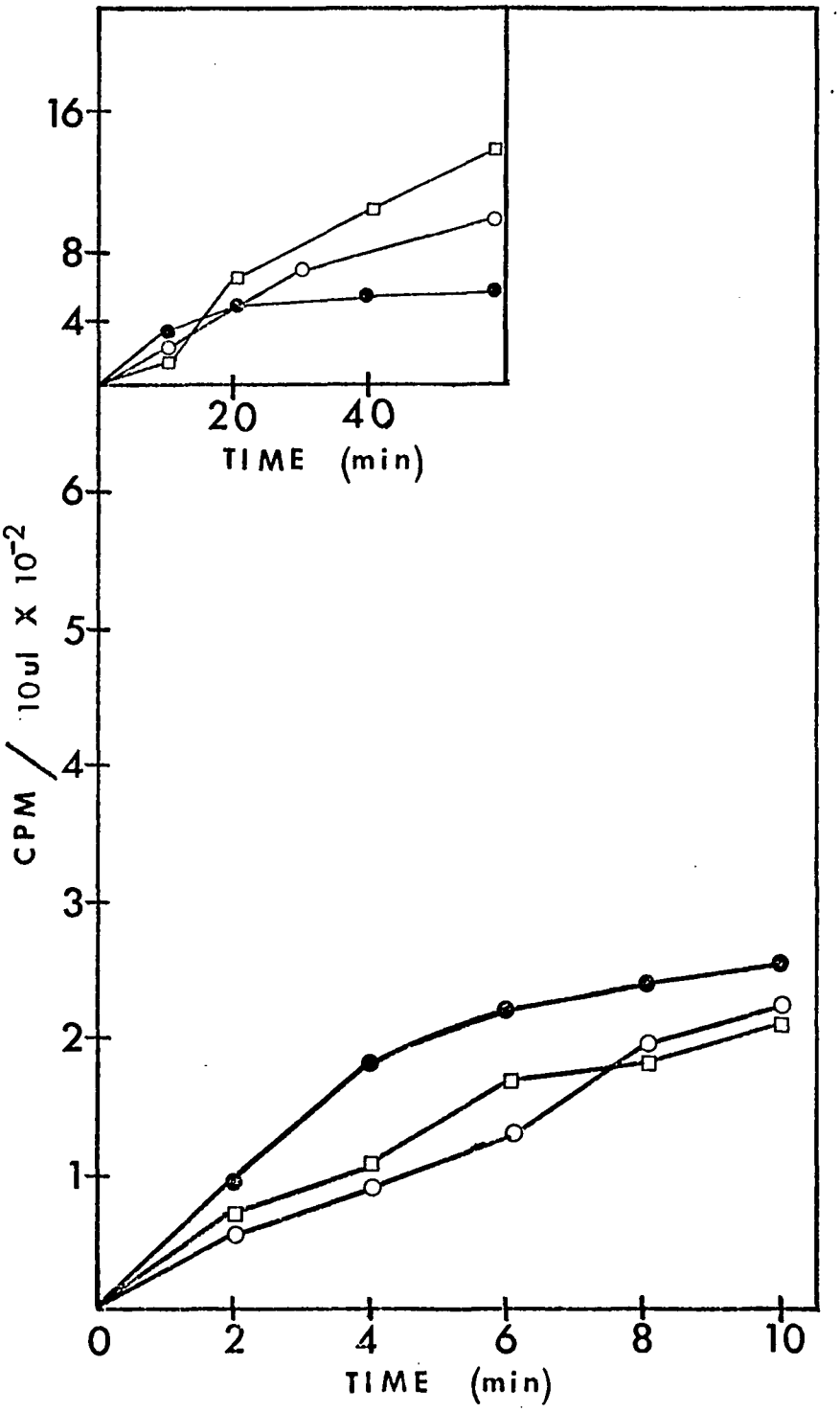
in the presence of 0.3 M KCl exhibits an early lag phase while spermidine in the presence of 0.1 M KCl although showing no lag, does induce little, if any, stimulation during the first ten minutes at this reduced temperature. The inset shows that synthesis in the presence of 0.1 M KCl is similar to that observed by the control reaction and that after ten minutes there is little difference in the rate of synthesis as influenced by 6 mM spermidine + 0.1 M KCl and 0.3 M KCl.

Figure 9 presents a comparison of the effect of 0.3 M KCl + 6 mM spermidine and 2.5 mM spermidine on the initial rates of RNA synthesis. Both conditions restrict synthetic activity slightly during the first ten minutes of incubation, however, some stimulation is observed after 30 minutes as shown in the inset. It must be remembered that the data obtained from assays incubated at 25° is not directly comparable to data obtained from an experiment carried out at the 37° temperature since the rate of synthesis is considerably reduced at the lower temperature. However, the kinetic behavior exhibited in Figures 8 and 9 do agree well with that expected from the results contained in Figure 7.

The combined data from Figures 7, 8 and 9 shows the absence of any large stimulatory response by either salt or spermidine in the early part of the RNA synthetic reaction. The overall and initial synthetic rates indicate that stimulation effected by KCl and spermidine becomes readily apparent only at about that time when the control reaction starts to plateau. Spermidine, especially appears to have little effect on RNA synthesis at either low, moderate, or high ionic strength conditions at the early time points in the reaction but is most effective after plateauing of the reaction is expected to take place.

FIGURE 9

Comparison of initial rates of RNA synthesis exhibited by reaction mixtures containing 0 KCl (●), 2.5 mM spermidine (O), and 6 mM spermidine + 0.3 M KCl (□). Reaction mixtures and assay conditions (25°) were as described for Figure 8.


16
8
4
20
40
TIME (min)
6
5
4
3
2
1
CPM / 10ul X 10^{-2}
0
2
4
6
8
10
TIME (min)

It can be argued that prior to the onset of plateauing, stimulation of RNA synthesis is due to either an increased rate of initiation or chain elongation or both, while once the plateau phase has been reached, the rates of initiation and elongation are of little importance if the polymerase is not free to reinitiate.

With this reasoning and because the spermidine effect is much more apparent at later times in the RNA synthetic reaction, it was felt that an investigation and comparison of the effects of spermidine and KCl on the termination events, including release of product RNA and reinitiation would be of value.

However, it is important to know if this stimulatory effect of KCl and spermidine is a universal phenomenon with respect to the DNA templates used or if it is peculiar to the T4 template. Table XIV presents the results of an experiment conducted to determine the effect of spermidine on transcription of 5 template preparations in the absence of KCl. Transcription of T4 DNA is stimulated approximately 3 fold by 2 mM spermidine while transcription of calf thymus DNA and T7 DNA are also stimulated about 3 fold, but at a spermidine concentration of 3 mM in the former case and 4 mM in the latter. Transcription of both E. coli DNA and poly [d(A-T)] is approximately doubled by spermidine at concentration of 3 mM and 8 mM respectively. It is interesting to note that precipitation, indicated by the asterisk, is observed at low spermidine concentration in reaction mixtures containing a very large molecular weight template, such as T4 DNA, and is not observed in reaction mixtures containing low molecular weight templates, such as poly [d(A-T)] and T7 DNA, even at relatively high concentrations of spermidine (8 mM).

TABLE XIV

Effect of Spermidine on Transcription of Several DNA Templates†

Spermidine (mM)	T4 DNA	C T DNA	E. coli DNA	poly [d(A-T)]	T7 DNA
	[^{3}H] UMP Incorporated: cpm/20 µl				
0	1,284	1,280	437	4,529	1,093
1	2,900	2,845	673	5,827	1,971
2	4,148	3,238	613	6,903	2,675
3	3,460	4,058	838	7,623	2,920
4	138*	3,386	633	7,446	4,043
6	176*	639*	580*	7,962	3,340
8	275*	264*	327*	8,414	---

* - Precipitate Observed in Assay Mixture

† The 0.2 ml reaction mixtures contained either 15 µg T4 DNA, 14.5 µg calf thymus DNA, 19 µg E. coli DNA, 14.4 µg Poly [d(A-T)] , 10.6 µg of T7 DNA and in all cases 2.5 µg of RNA polymerase. Time of incubation was 180 minutes. C T = calf thymus.

Table XV shows the effect of KCl on the transcription of these same templates. It is immediately noticed that KCl is markedly more effective in stimulating transcription of T4 and T7 templates showing some 6 - 7 fold stimulation by 180 minutes of incubation. Transcription of calf thymus DNA, E. coli DNA and poly [d(A-T)] is stimulated to a lesser degree by high concentrations of KCl than by low concentrations of spermidine.

TABLE XV

Effect of KCl on Transcription of Several DNA Templates*

Concentration of KCl (M)	T4 DNA	C T DNA	E. coli DNA	poly [d(A-T)]	T7 DNA
		[^{3}H] UMP Incorporated:		cpm/20 µl	
0.00	1,482	1,500	223	3,403	1,361
0.10	1,732	2,850	261	5,291	---
0.20	5,500	3,384	344	6,622	6,027
0.25	8,656	3,576	365	6,914	6,374
0.30	10,628	2,894	378	5,443	3,912
0.40	804	830	250	2,035	---

* Reaction mixtures were as described in Table XIV.

Since data from Table XV indicated that KCl elicited very little stimulation of calf thymus DNA transcription, it was considered of interest to investigate the influence of spermidine on transcription of calf thymus DNA at intermediate (0.1 M KCl) and high (0.3 M KCl) KCl concentrations. As shown in Table XVI, spermidine does stimulate transcription of T4 DNA at both salt conditions but, under the same conditions, is very much less effective in stimulating calf thymus DNA transcription. A comparison of Table XIV and XVI reveals that spermidine stimulates RNA synthesis some 3 fold at both conditions of 0 KCl and 0.1 M KCl, although requiring a slightly greater concentration in the latter case. Essentially no stimulation of calf thymus DNA transcription by spermidine is observed in the presence of 0.3 M KCl. It appears, then, that spermidine in the presence of high salt concentrations, as in the case of 0.3 M KCl alone, is not highly effective in increasing transcription of calf thymus

DNA; although spermidine, in the absence of KCl or at an intermediate KCl concentration, is moderately effective. The nature of the template does, therefore, play a role in cation-mediated stimulation of *in vitro* transcription.

TABLE XVI

Comparative Effect of Spermidine on Transcription of T4 and Calf Thymus DNA at Intermediate (0.1 M KCl) and High (0.3 M KCl) Salt Conditions*

Concentration of Spermidine (mM)	Concentration of KCl (M)	Template	^{14}C-cpm/20 µl
0	0.1	T4	2,177
4	0.1	T4	12,209
6	0.1	T4	13,923
8	0.1	T4	14,912
0	0.3	T4	10,328
4	0.3	T4	13,387
6	0.3	T4	15,766
8	0.3	T4	14,886
0	0.1	CT	1,309
4	0.1	CT	3,288
6	0.1	CT	4,069
8	0.1	CT	3,961
0	0.3	CT	2,314
4	0.3	CT	2,813
6	0.3	CT	3,008
8	0.3	CT	2,789

* The reaction mixture contained 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 1 mM ATP, GTP, and CTP; 0.5 mM ^{14}C-UTP (4 x 10^5 dpm); 0.1 mM DTT; 12 µg T4 DNA or 15 µg calf thymus DNA; 5 µg RNA polymerase; and the amount of KCl or spermidine indicated. The incubation time was 120 minutes. CT = calf thymus.

Bremer and Konrad (94) reported, almost a decade ago, that RNA molecules synthesized in vitro under low ionic strength conditions remain complexed with the template and polymerase resulting in an RNA-enzyme-DNA complex sufficiently stable to survive sedimentation through a sucrose gradient. They noted, however, that if the reaction was conducted at high ionic strength, release of the nascent RNA was observed. Richardson (23), using the nitrocellulose membrane filter method, developed by Jones and Berg (37), reported that prior to 30 minutes of incubation, at low ionic strength, all the newly synthesized RNA remained complexed, but after 30 minutes, the amount of complex bound RNA decreased until, at 180 minutes, only 50% of the total RNA existed in the complexed form. In 1970 Millette and Trotter (100) reported a large scale release of nascent RNA from the transcription complex even at low salt conditions. More recently, Witmer (89) reported data confirming the earlier work of Bremer and Konrad.

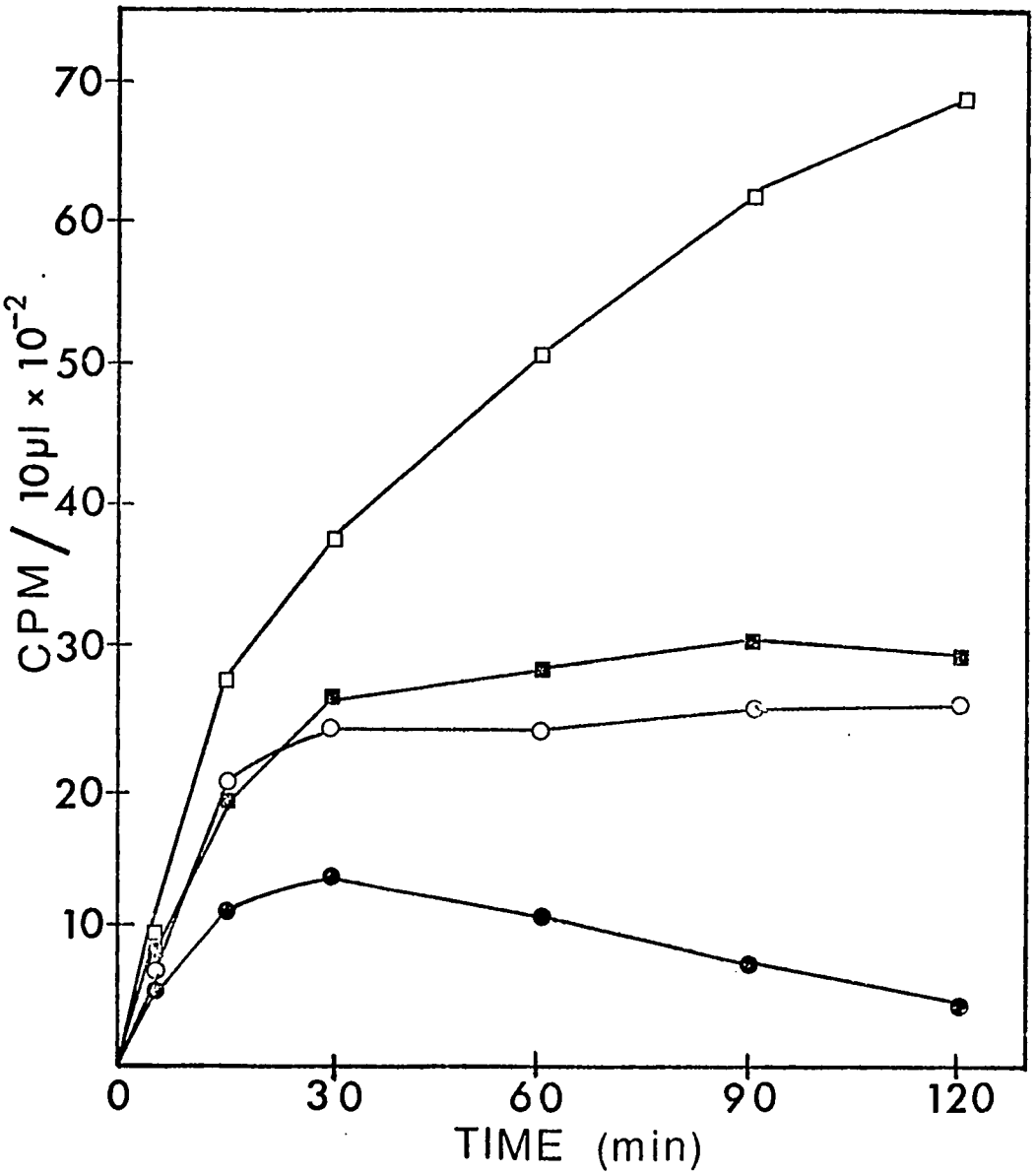
Since KCl had previously been reported to facilitate release of RNA from the transcription complex, it was felt that spermidine also would allow release thus accounting for the observed spermidine-mediated stimulation of RNA synthesis.

The nitrocellulose membrane filter method, in which complex-bound RNA is retained by the membrane filter while free RNA is not, (123) was used to test this theory. By determining the total RNA synthesized by precipitation with TCA, as mentioned in the Methods section, and comparing that value with the amount of RNA retained by the membrane filter, an estimate of the amount of free RNA was made.

Figure 10 shows that the results of membrane filter analysis of reaction mixtures incubated for 120 minutes in the presence and absence of 2.5 mM spermidine in the absence of KCl. In the absence of spermidine,

FIGURE 10

Determination of total and bound RNA synthesized in the presence and absence of 2.5 mM spermidine by the nitrocellulose membrane filter analysis: the reaction mixtures contained 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 1 mM ATP, CTP, and GTP, 0.5 mM ^{3}H-UTP (1.8×10^6 dpm); 0.1 mM DTT; 15 µg ^{32}P-T4 DNA (1,500 dpm/µg); 2.5 µg RNA polymerase. Incubation temperature was 37°. A detailed discussion of filter analysis is presented in the Methods section. (O) total ^{3}H-cpm incorporated (minus spermidine), (●) ^{3}H-cpm retained by membrane filter (minus spermidine), (□) total ^{3}H-cpm incorporated (plus spermidine), (■) ^{3}H-cpm retained by membrane filter (plus spermidine).


70
60
50
40
30
20
10
CPM / 10µl x 10^{-2}
0
30
60
90
120
TIME (min)

the total incorporated ^{3}H-UMP counts reached a plateau between 15 and 30 minutes, and remained constant for the remainder of the two hour incubation period. Only 50% of the total incorporated ^{3}H-UMP was retained by the membrane filter at 60 minutes, decreasing to 25% by 120 minutes. This suggests that 50% of the RNA was released by 60 minutes and that as much as 75% of the total RNA was free RNA at 120 minutes. With 2.5 mM spermidine, no plateau was observed and the amount of label retained by the membrane filter reached a maximum at about 30 minutes and remained constant for the remaining 90 minutes, indicating that much of the RNA was released under these conditions as well. These results are unexpected, in that complex-bound RNA appears to be released even at early times at low ionic strength conditions. It is interesting to note that the amount of complex-bound RNA observed in the presence of 2.5 mM spermidine is the same as the total amount of RNA synthesized in the absence of spermidine. This indicates that transcription stops after one round of synthesis at low ionic strength since the total amount of RNA synthesized in the absence of spermidine is equal to the maximum amount of RNA present in an active transcription complex found in the presence of spermidine; a situation where early plateauing is not observed.

^{32}P-labeled T4 DNA was used as template in these experiments in order to provide an indirect method of observing the release of the RNA polymerase from the transcription complex. Jones and Berg (37) had previously reported that neither RNA polymerase nor DNA was retained separately by nitrocellulose filters but, that they were retained if complexed with each other. Furthermore, Freeman and Jones (121) had suggested that the binding of at least 5 RNA polymerase molecules to one DNA molecule was required before the complex was retained by the membrane

filter. Therefore, it followed that if the RNA polymerase is released from the DNA template, a reduction in ^{32}P-label retained by the membrane filter would result. No such reduction in filter-bound ^{32}P-counts was observed in these experiments. In fact, the amount of ^{32}P-label retained by the membrane filter was equal to the total TCA-precipitable ^{32}P-counts throughout the entire reaction period.

The data were interpreted as indicating that at low ionic strength conditions, in the absence of reinitiation but with release of nascent RNA, the polymerase is not released but remains with the DNA. However, this interpretation may not be valid in the light of a recent report by Hinkle and Chamberlin (38) indicating that the polymerase by itself sticks to the membrane filter and that only one polymerase per DNA molecule is required for retention of DNA by the filter. In light of these results, interpretation of the ^{32}P-retention data is made difficult.

One might ask whether spermidine has any effect upon the tendency of the membrane filter to retain ^{3}H-RNA. Table XVII shows that when spermidine was added to one half of a low salt reaction mixture to a concentration of 2.5 mM and the total TCA-precipitable and filter-bound RNA was determined for both halves of the reaction mixture, no difference in binding was observed. Thus, spermidine appears to have no effect upon determination of either total or complex-bound RNA.

Since the membrane filter analysis yielded unexpected results, sucrose gradient analysis of the reaction mixtures were performed. Low ionic strength reaction mixtures, with and without spermidine, and containing ^{32}P-labeled T4 DNA were incubated for 5, 30, or 60 minutes and layered onto 5-20% sucrose gradients. Figure 11 shows the sedimentation

profiles of the reaction mixtures.

TABLE XVII

Effect of Spermidine on Total and Complex Bound RNA Determination*

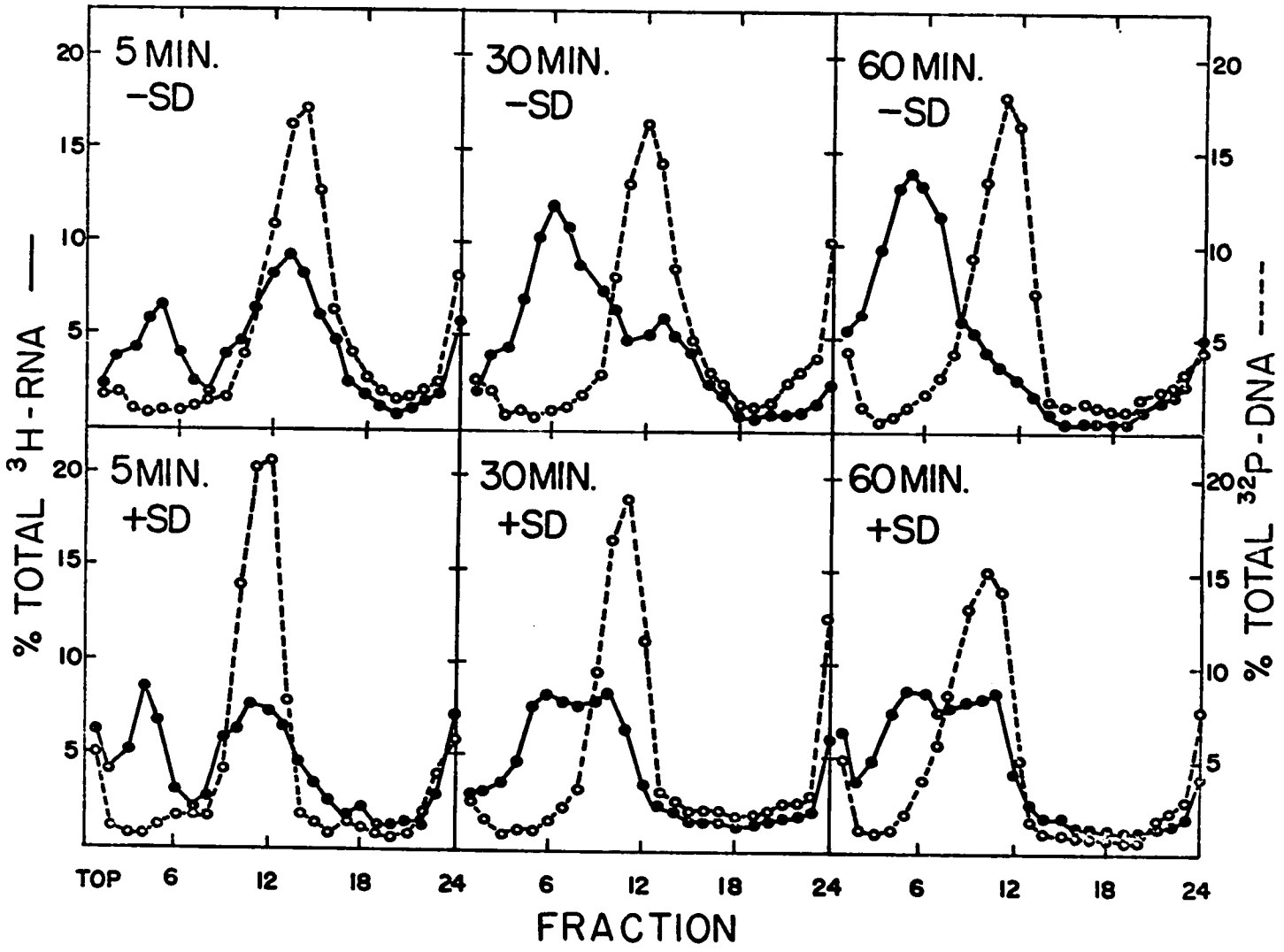
Concentration of Spermidine (mM)	Total ^{3}H cpm/ 10 µl - blank	Filter Bound ^{3}H cpm/ 10 µl - blank
0	1,050	264
2.5	1,021	245

* The low salt reaction mixtures and assay conditions were as described for Figure 10. At the end of a 120 minute incubation period, the reaction mixture was divided in half and spermidine was added to one half to a concentration of 2.5 mM. Total TCA-precipitable and membrane filter retained ^{3}H incorporated counts were determined for both halves from 10 µl aliquots as described in the Methods section.

The sedimentation profiles of the 5 minutes reaction mixtures with and without spermidine revealed similar ^{3}H-RNA peaks. The free RNA appeared as a peak near the top of the gradient while the complex-bound RNA sedimented with the ^{32}P-T4 DNA as a peak in the middle of the gradient. At 30 minutes the proportion of free RNA to complex-bound RNA increased in the reaction mixture lacking spermidine and nearly all RNA was released by 60 minutes. In the presence of spermidine, however, the proportion of free to bound RNA increased only slightly from 30 to 60 minutes while the total amount of RNA synthesized increased substantially (see Figure 11 legend). These results indicate that an active transcription complex is present throughout the 60 minute incubation period in reaction mixtures containing spermidine but not in reaction mixtures lacking spermidine. The combined results contained in Figures 10 and 11 suggest that reinitia-

FIGURE 11

Sucrose gradient analysis of reaction mixtures (±) spermidine at 5, 30, and 60 minutes of incubation. Reaction mixtures and assay conditions were as described for Figure 10. (O) ^{32}P-T4 DNA (⊗) ^{3}H-RNA. A detailed discussion of the procedure for sucrose gradient analysis is presented in the Methods section. Count data for the reaction mixtures were as follows: low salt 5, 30, and 60 minutes = 729 cpm/10 µl, 2,486 cpm/10 µl, and 2,673 cpm/10 µl respectively; 2.5 mM spermidine, 5, 30, and 60 minutes = 842 cpm/10 µl, 2,716 cpm/10 µl and 3,884 cpm/10 µl respectively.


5 MIN.
-SD
30 MIN.
-SD
60 MIN.
-SD
5 MIN.
+SD
30 MIN.
+SD
60 MIN.
+SD
% TOTAL ^{3}H-RNA ——
% TOTAL ^{32}P-DNA ----
TOP
6
12
18
24
FRACTION

tion occurs at low ionic strength in the presence of spermidine but not in its absence.

The percent recovery of the ^{3}H-RNA layered on the sucrose gradients was usually 85-95% and always greater than 70%. 100% of the ^{32}P-DNA was always recovered. Also, the presence of Mg^{++} in the gradient buffer over a concentration range of 0 to 4 mM had no effect upon percent recovery, sedimentation behavior of polynucleotides, or relative proportion of free and complex-bound RNA.

To further test these results, both membrane filter and sucrose gradient analysis were performed on the same reaction mixtures. Table XVIII presents a comparison of data obtained from analysis of reaction mixtures with and without spermidine. For reaction mixtures lacking spermidine the membrane filter analysis indicates an increase in the proportion of free RNA from 19% at 5 minutes to 62% at 60 minutes of incubation while showing a decrease from 81% to 38% of the complex-bound RNA over the same time period. Analysis by sucrose gradient is in good agreement, showing an increase of free RNA from 29% to 78% and a decrease of bound RNA from 57% to 20%. The data are in better agreement if the ^{3}H-counts present in the pellet are considered to be complex-bound RNA. That this is probably the case is suggested by the presence of ^{32}P-counts in the pellet.

In reaction mixtures containing spermidine, the proportion of free to bound RNA increases from 36% free RNA at 5 minutes to 50% at 60 minutes when analyzed by the filter method. The complex-bound RNA decreases from 64% to 50%. Gradient analysis indicates an increase from 35% to 51% for free RNA and a decrease of 50% to 43% for complex-bound RNA.

TABLE XVIII

Comparison of Nitrocellulose Filter Data and Sucrose Gradient Data*

	Free RNA		Bound RNA		Pellet	
	(-)SD	(+)SD	(-)SD	(+)SD	(-)SD	(+)SD
5 minutes						
Gradient	29%	35%	57%	50%	11%	13%
Filter	19%	36%	81%	64%	---	---
30 minutes						
Gradient	63%	42%	31%	46%	5%	10%
Filter	52%	36%	48%	64%	---	---
60 minutes						
Gradient	78%	51%	20%	43%	1%	5%
Filter	62%	50%	38%	50%	---	---

* The percentage of free and bound RNA observed in sucrose gradients was determined by calculating the number of counts represented by the area under the free and bound RNA peaks and dividing by the total recovered ^{3}H-RNA counts. The percent of free RNA was determined from the membrane filter method by subtracting ^{3}H-counts retained by the filter from the total acid percipitable ^{3}H-counts divided by total ^{3}H-counts.

The combined data strongly suggest that early cessation of synthesis is not a result of the formation of a stable RNA-enzyme-DNA complex due to the failure of the nascent RNA to be released. Even at low ionic strength, where reinitiation does not occur, newly synthesized RNA is released. Spermidine, therefore, does not stimulate RNA synthesis by facilitating the release of newly synthesized RNA from the transcription complex. The data do not, however, challenge the theory that inhibition by product RNA is responsible for failure to reinitiate.

The size of the product RNA has been reported by some investigators (79, 104, 122) to be influenced by the ionic strength environment of the RNA synthetic reaction mixture. To investigate the possibility that spermidine and KCl influence the size of the RNA synthesized, reaction mixtures containing KCl or spermidine were subjected to analysis on SDS-sucrose gradients (see Methods). The Spinco SW 27.1 rotor was selected for the analysis because the large length to diameter ratio improves resolution of macromolecules in gradients.

Figure 12 shows the gradient profiles exhibited by the RNA products from low salt, 2.5 mM spermidine, and high salt reaction mixtures after a 90 minute incubation period. Four peaks are observed possessing sedimentation coefficients ranging from just under 23 S to just over 35 S. Count data, as cpm/10 µl aliquots removed at the end of the 90 minute incubation period, are presented to show that KCl and spermidine-mediated stimulation did occur in reaction mixtures analyzed by the SDS-sucrose gradients. The only observable differences between the gradient profiles lie near the top of the gradient where a larger percentage of small molecular weight RNA is present in the low salt and spermidine reactions than in the KCl reaction. There is no evidence that larger molecular weight RNA transcripts are synthesized in the presence of spermidine or KCl. Therefore, KCl and spermidine do not effect the size of the in vitro T4 transcripts.

To obtain the molecular weights of the four main RNA species observed in the SDS-sucrose gradients, the reaction mixtures were subjected to centrifugation on formaldehyde gradients (see Methods) after the method of Boedtker (97). Figure 13 shows that the four major RNA species possess molecular weights ranging from 1×10^6 to 2.9×10^6,

FIGURE 12

SDS-sucrose gradient analysis of 0 KCl (Δ), 0.3 M KCl (O), and 2.5 mM spermidine (●) reaction mixtures. The reaction mixtures and assay conditions were as described for Figures 10 and 11. 10 µl aliquots were removed at the end of the 90 minute incubation period for determination of total ^{3}H-UMP incorporation. The cpm/10 µl are reported in the inset. The remaining reaction mixtures were treated as described in the Methods section followed by centrifugation of the 15 ml SDS (5-30%) sucrose gradients in a Spinco SW 27.1 rotor at 80,900 x G for 10.5 hours at 22°. 0.5 ml fractions were collected. 16 S and 23 S rRNA was added as internal markers.

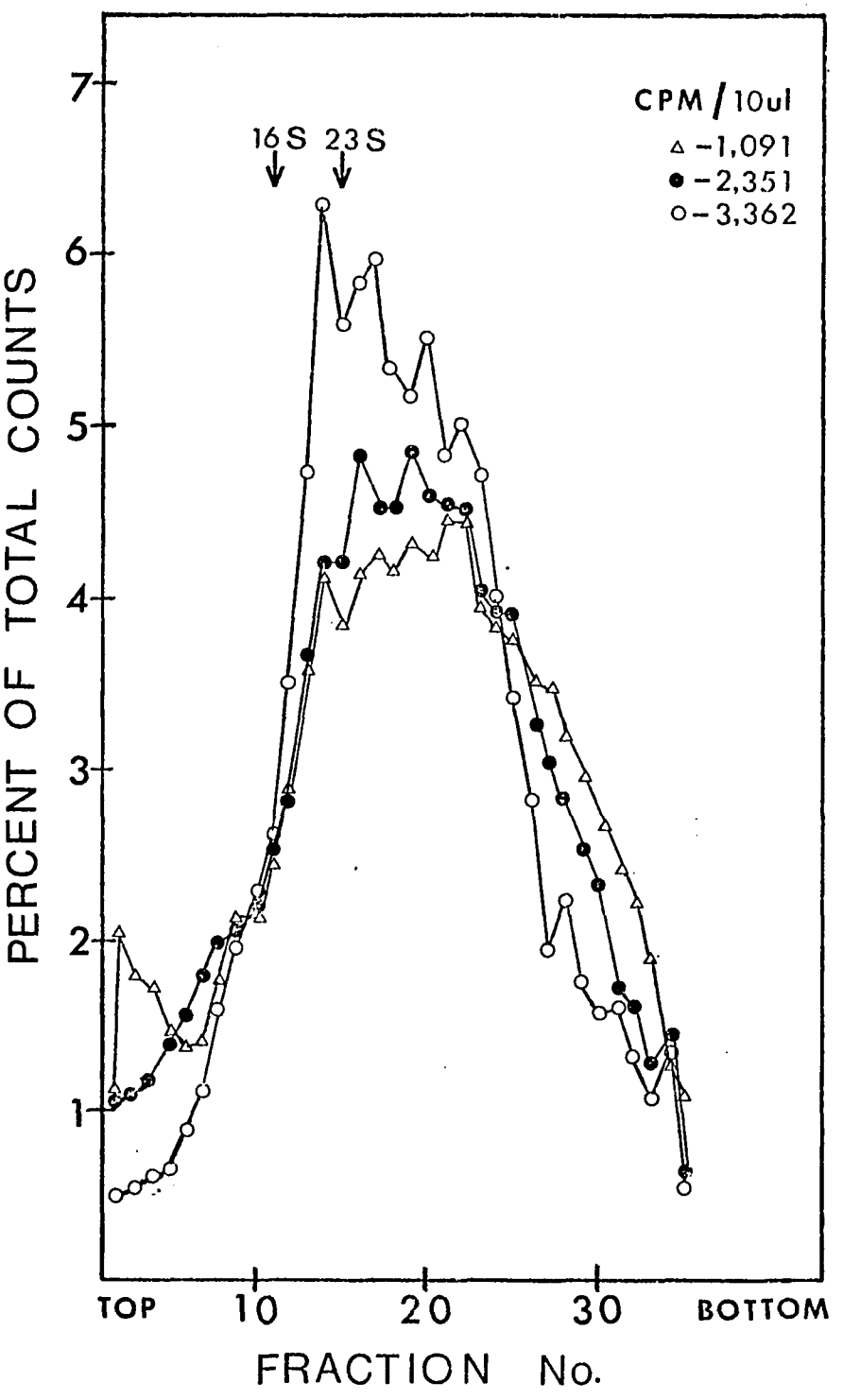
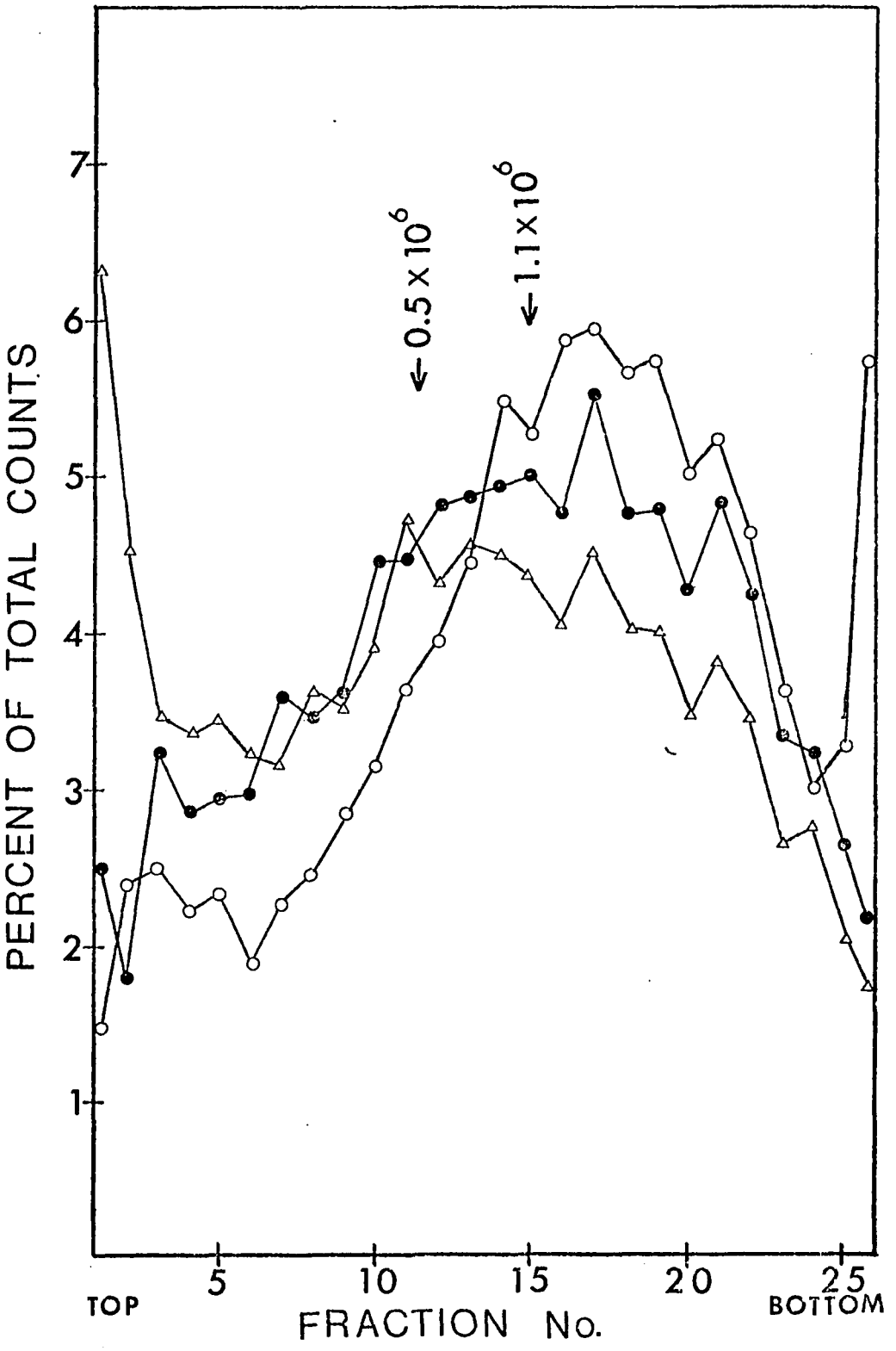

CPM / 10ul
△ - 1,091
● - 2,351
○ - 3,362
16S
23S
PERCENT OF TOTAL COUNTS
7
6
5
4
3
2
1
TOP
10
20
30
BOTTOM
FRACTION No.

FIGURE 13

Analysis of in vitro T4 transcripts by formaldehyde-sucrose gradients. Reaction mixtures and assay conditions were as described for Figure 10. 10 µl aliquots were removed at 60 minutes for determination of total ^{3}H-UMP incorporation. The molecular weights shown are according to Boedtker (97) for 16 S and 23 S E. coli rRNA used as markers. A detailed discussion of the procedure followed for formaldehyde-sucrose gradient analysis is presented in the Methods section. (Δ) 0 KCl, (●) 2.5 mM spermidine, (O) 0.3 M KCl. Total ^{3}H-UMP incorporation reported as cpm/10 µl for the three reaction conditions was: 0 KCl = 554 cpm/10 µl, 2.5 mM spermidine = 976 cpm/10 µl, 0.3 M KCl = 1,154 cpm/10 µl.


PERCENT OF TOTAL COUNTS
7
6
5
4
3
2
1
← 0.5 X 10^6
← 1.1 X 10^6
5
10
15
20
25
TOP
BOTTOM
FRACTION No.

calculated according to Boedtker, corresponding to a range in nucleotide chain lengths of 2,881 to 8,357 nucleotides. Both Millette and Trotter (100) and Witmer (89) reported two main species of T4 product RNA with average chain lengths of 4,300-5,000 nucleotides for one and 7,100-7,500 nucleotides for the other. This agrees well with the chain lengths calculated for an average of the first two and second two major peaks observed in Figure 13: 4,034 and 7,492 nucleotides respectively. Again there is no major difference in the profiles exhibited by low salt, spermidine, or high salt reaction mixtures.

Richardson (104) earlier showed that in the presence of 0.2 M KCl, RNA polymerase is released from the template on which it initiated transcription and is able to reinitiate new RNA chain synthesis on a new template. That is, under conditions where reinitiation does occur, the enzyme dissociates from the template before initiating new RNA chains. This was shown by first allowing initiation on T4 DNA followed by the addition of T5 DNA after 10 minutes of incubation. The reaction was terminated 10 minutes later and the product RNA was hybridized against the T4 and T5 DNA templates. The product RNA synthesized at low ionic strength conditions hybridized only to the T4 DNA. Product RNA from reaction mixtures containing 0.2 M KCl, however, also hybridized to the T5 or second template showing that the polymerase had dissociated from the first template. These results do not, however, rule out the possibility that the polymerase is also able to dissociate from the template at low salt conditions but is unable to reinitiate because of interaction with product RNA or some other reversible inactivation event preventing reinitiation.

It was expected that the presence of spermidine, at concentrations which would not appreciably alter the ionic strength of the assay medium, would also result in reinitiation by the polymerase on a new template. Experiments similar to those reported by Richardson were performed but with calf thymus and T4 DNA. The use of calf thymus DNA was deliberate since little salt stimulation is observed with this template (Table XV) and because core enzyme is quite efficient in transcribing it (33). This was useful because it was thought that perhaps the inability to reinitiate on either T4 or T5 template at low salt conditions was due to a reversible inactivation of sigma factor. If this were the case, the addition of calf thymus DNA as the second template would result in its transcription by core enzyme even at low salt conditions.

Table XIX presents the results of these experiments. It can be seen that when calf thymus DNA and T4 DNA are present from the beginning of the reaction, transcription of calf thymus DNA is preferred at low ionic strength and in the presence of spermidine. With 0.3 M KCl present, however, transcription of T4 DNA is greatly preferred. This result is to be expected from the differential stimulatory response elicited by KCl with T4 and calf thymus DNA as previously shown in Table XV. When T4 DNA is added as the challenger template, only 5 percent of the product RNA is T4 specific at low salt conditions. The presence of 2.5 mM spermidine results in a 5 fold increase in the amount of T4 specific transcript produced. 0.3 M KCl in the reaction mixture, as expected, also increases the amount of product RNA synthesized from the second template. The unusually large increase observed can be explained by considering the large preference for T4 transcription exhibited in reaction mixtures containing 0.3 M KCl. When calf thymus DNA is the

challenger DNA, a 4 to 5 fold increase in transcription of the second template is observed with either KCl or spermidine in the reaction mixture. It is noticed that at low salt conditions, calf thymus DNA is not an efficient challenger indicating that an inactivation of sigma is not responsible for failure to reinitiate but that the core enzyme is, in some way, reversibly inactivated under these ionic conditions.

TABLE XIX

Reinitiation

Conditions	Initial Template	Challenger Template	^{3}H-UMP Incorporated After Addition of Second Template	Fraction of RNA Made After Addition of Second Template:	
			cpm/10 µl	CT %	T4
Control	CT + T4	--	1,422	68	32
2.5 mM SD	CT + T4	--	2,832	64	36
0.3 M KCl	CT + T4	--	3,372	15	85
Control	CT	T4	658	95	5
2.5 mM SD	CT	T4	1,302	75	25
0.3 M KCl	CT	T4	1,525	12	88
Control	T4	CT	927	4	96
2.5 mM SD	T4	CT	1,653	18	82
0.3 M KCl	T4	CT	1,258	21	79

* Each 0.2 ml reaction mixture initially contained 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 0.2 mM ATP, CTP, and GTP; 0.05 mM UTP; 0.1 mM DTT, 12 µg of either T4 or calf thymus DNA; and 2.5 µg of polymerase. After 20 minutes of incubation at 37°, 16 µg of a second DNA and 9.4 x 10^5 dpm of ^{3}H-UTP were added. Synthesis was continued for an additional 60 minutes at which time a 10 µl aliquot was removed for determination of total ^{3}H-UMP incorporation. Purification of RNA and actual hybridization followed, as described in Methods. The amount of labeled RNA remaining on the DNA filters after hybridization was normalized by first subtracting the counts on the blank filter and then dividing by the efficiency of hybridization determined for pure preparation of T4 and calf thymus DNA (T4 = 0.25, CT = 0.068). The fraction of RNA specific for each template is reported as percent of total. In each case the total of the normalized value was within 15 percent of the total amount of RNA added to the hybridization cocktails. SD = spermidine, CT = calf thymus.

Several factors must be taken into consideration when interpreting these data. First, it must be realized that ^{3}H-UTP is not added to the reaction until the time of addition of the second template. Therefore, only cold product RNA is made during the first 20 minutes of incubation. This results in competition between labeled product made after the addition of ^{3}H-UTP and unlabeled RNA made prior to this time point for hybridization with the first template. No such competition occurs for hybridization with the second template since only labeled product is made from that template. Therefore, the presence of a considerable amount of cold calf thymus DNA product, competing with labeled calf thymus product, in conjunction with the preferential transcription of T4 DNA, resulting in a large amount of "labeled only" T4 product RNA, would explain the hybridization results observed in the case where T4 DNA is the challenging template, in the presence of 0.3 M KCl. The presence of spermidine would not give rise to these results since 2.5 mM spermidine is less stimulatory in the early part of the reaction than KCl and spermidine does not cause the preferential stimulation of T4 DNA transcription.

These considerations do not invalidate the conclusions that a significantly larger number of polymerase molecules dissociate from the initial template and are able to transcribe a second "challenger" template in the presence of 2.5 mM spermidine or 0.3 M KCl than at low salt conditions, in the absence of spermidine.

Since both spermidine and KCl appeared to prevent early cessation of RNA synthesis, it was considered of interest to see if these compounds could restart synthesis once the plateau was established. The experiment was conducted in the following manner: all tubes containing 0.1 M

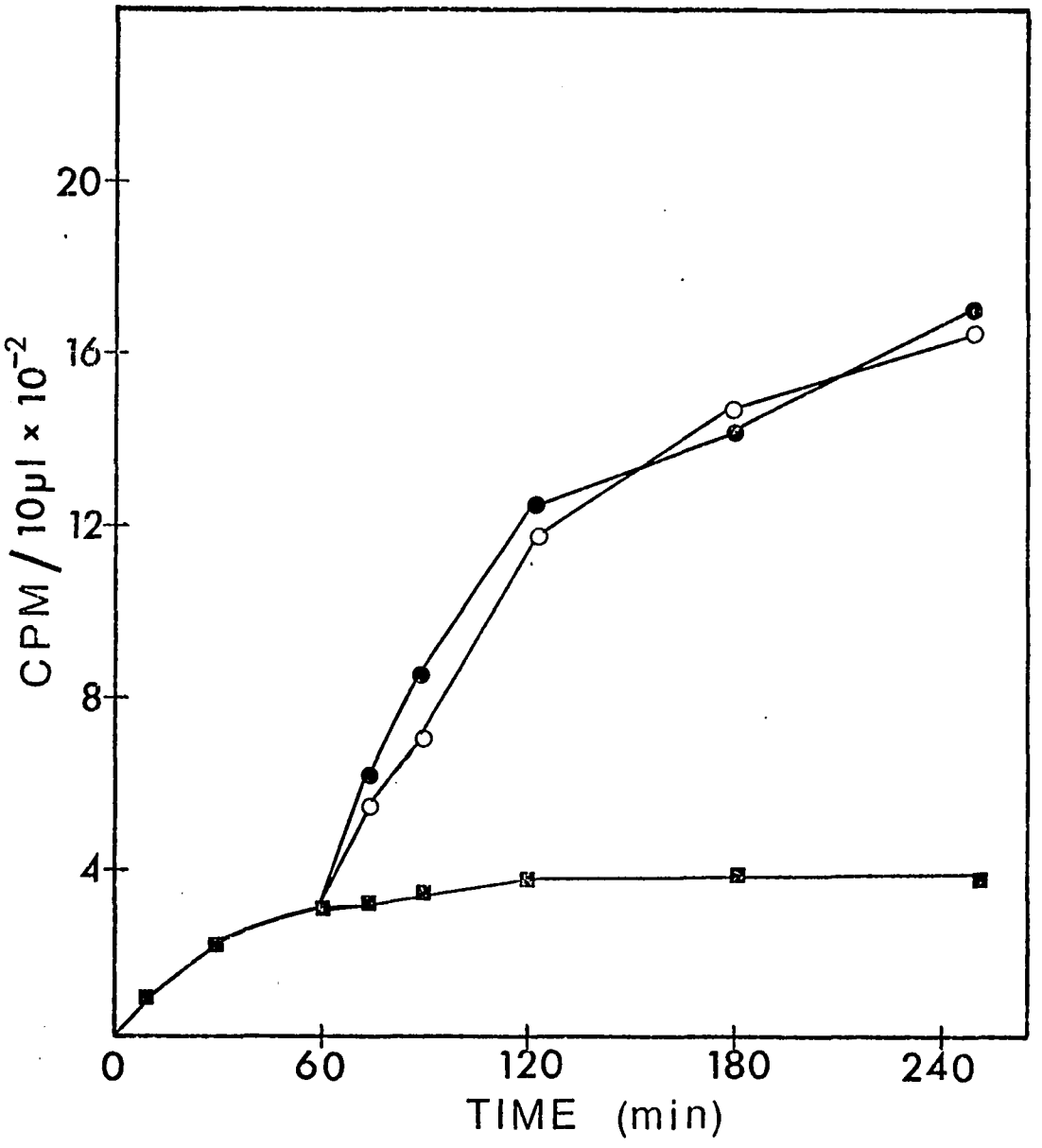
KCl were incubated for 60 minutes at which time 0.2 ml of 60 mM spermidine, 2 M KCl, or distilled water were added to tubes yielding reaction mixtures containing 0.1 M KCl + 6 mM spermidine, 0.3 M KCl, or 0.1 M KCl. The results, presented in Figure 14, shows that both 0.3 M KCl and 6 mM spermidine are very effective in immediately stimulating reinitiation of RNA synthesis, resulting in a 3 fold stimulation at 180 minutes. Again 6 mM spermidine + 0.1 M KCl and 0.3 M KCl alone appear equally effective in stimulating RNA synthesis. These experiments were conducted with 0.1 M KCl in the reaction mixture to prevent precipitation of the system by spermidine.

If the cessation of RNA synthesis observed at low ionic strength conditions is due to product inhibition by RNA, then the addition of RNA to an assay mixture should inhibit RNA synthesis from the beginning of the assay. Table XX shows that the addition of either E. coli tRNA (stripped) or E. coli 16 S and 23 S ribosomal RNA to the reaction mixture, prior to initiation, results in inhibition. Furthermore, greater inhibition is observed when RNA is preincubated with the polymerase than when the reaction is started by addition of the enzyme to a reaction mixture containing both DNA and RNA, indicating that there is a competition between the DNA and RNA for the polymerase.

Spermidine and KCl reduce, to a large measure, the inhibition of synthesis by RNA. Table XXI shows that when RNA is preincubated with the polymerase, in a reaction mixture containing 0.05 M KCl, 0.05 M KCl + 6 mM spermidine, or 0.3 M KCl, followed by addition of DNA to start the reaction, the amount of inhibition is reduced in reaction mixtures containing spermidine or 0.3 M KCl. Spermidine is somewhat more effective

FIGURE 14

Restart experiment: the 0.2 ml reaction mixtures originally contained 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 1 mM ATP, CTP, and GTP; 0.5 mM ^{3}H-UTP (1.8×10^6 dpm); 0.1 mM DTT, 0.1 M KCl; 15 µg T4 DNA; and 5 µg RNA polymerase. At 60 minutes, 0.2 ml of either 60 mM spermidine, 2 M KCl, or water was added to separate tubes yielding reaction mixtures containing 0.1 M KCl + 6 mM spermidine, 0.3 M KCl, or 0.1 M KCl. Incubation continued for an additional 180 minutes. 10 µl aliquots were removed from each reaction tube at the time points indicated with sample preparation for counting as described in the Methods section. (▣) 0.1 M KCl control; (●) 0.1 M KCl + 6 mM spermidine, (O) 0.3 M KCl.


20
16
12
8
4
CPM / 10µl × 10⁻²
0
60
120
180
240
TIME (min)

than KCl in preventing RNA inhibition especially in the case where E. coli tRNA is added.

TABLE XX

Effects of the Order of Addition of RNA Polymerase and Template on Inhibition by RNA*

E. coli tRNA (stripped)					E. coli 16 S and 23 S rRNA				
RNA added	added after preincubation		cpm/ 10µl	% inhibition	RNA added	added after preincubation		cpm/ 10µl	% inhibition
	DNA	Enz				DNA	Enz		
0	X		589	0	0	X		475	0
20µg	X		461	21.7	10µg	X		287	39.5
40µg	X		292	50.4	20µg	X		106	77.6
60µg	X		235	60.1	40µg	X		26	94.5
80µg	X		176	70.1					
0		X	842	0	0		X	575	0
20µg		X	792	6	10µg		X	375	34.7
40µg		X	584	31	20µg		X	434	24.5
60µg		X	583	31	40µg		X	370	35.6
80µg		X	497	41					

* The final 0.2 ml reaction mixture contained 50 mM Tris-HCl, pH 7.9; 5 mM $MgCl_2$; 0.2 mM GTP, CTP, and UTP; 0.2 mM ^{14}C-ATP ($9x10^4$ dpm); 0.1 mM DTT, 0.05 M KCl; 14µg T4 DNA; 5µg RNA Polymerase; and E. coli tRNA (stripped) or E. coli 16 S and 23 S ribosomal RNA mixture. The reaction was started by the addition of either DNA or polymerase as indicated, after a 5 minute preincubation at room temperature of the reaction mixture lacking DNA or polymerase. 10µl aliquots were removed 50 minutes after addition of last reaction component and prepared for counting as described in the methods.

Table XXII shows the comparative effect of KCl and spermidine in overcoming inhibition. In this experiment RNA was again preincubated with the polymerase, but spermidine and KCl were not added until after the preincubation period of 5 minutes, i.e., at the time of the addition of DNA. Again spermidine appears to be more effective in overcoming RNA inhibition by tRNA, but both spermidine and KCl are equally effective in partially overcoming inhibition by 16 S and 23 S rRNA.

TABLE XXI

Effects of Spermidine and KCl in Preventing Inhibition by RNA

E. coli tRNA (stripped)					E. coli 16 S and 23 S rRNA				
amount of RNA added to 0.2 ml assay	concentration of KCl	concentration of spermidine	cpm/ 10µl blank	% inhibition	amount of RNA added to 0.2 ml assay	concentration of KCl	concentration of spermidine	cpm/ 10µl blank	% inhibition
0	0.05M	---	712	0	0	0.05M	---	462	0
40µg	0.05M	---	416	35	20µg	0.05M	---	104	77
60µg	0.05M	---	289	59	40µg	0.05M	---	43	91
0	0.05M	6mM	1,773	0	0	0.05M	6mM	1,435	0
40µg	0.05M	6mM	1,708	4	20µg	0.05M	6mM	1,170	18
60µg	0.05M	6mM	1,701	4	40µg	0.05M	6mM	1,123	22
0	0.3M	---	1,733	0	0	0.3M	---	1,263	0
40µg	0.3M	---	1,484	14	20µg	0.3M	---	1,033	18
60µg	0.3M	---	1,103	36	40µg	0.3M	---	672	47

The final 0.2 ml reaction mixtures were as described for Table XX. 6 mM spermidine and 0.3 M KCl were present during the preincubation period. The reaction was started by addition of DNA.

TABLE XXII

Effects of Spermidine and KCl in Overcoming Inhibition by RNA

E. coli tRNA (stripped)					E. coli 16 S and 23 S rRNA				
amount of RNA added to 0.2 ml assay	concentration of KCl	concentration of spermidine	cpm/ 10µl blank	% inhibition	amount of RNA added to 0.2 ml assay	concentration of KCl	concentration of spermidine	cpm/ 10µl blank	% inhibition
0	0.05M	---	675	0	0	0.05M	---	552	0
40µg	0.05M	---	423	37	20µg	0.05M	---	202	63
60µg	0.05M	---	238	65	40µg	0.05M	---	52	91
0	0.05M	6mM	1,512	0	0	0.05M	6mM	1,519	0
40µg	0.05M	6mM	1,292	15	20µg	0.05M	6mM	1,196	21
60µg	0.05M	6mM	1,374	9	40µg	0.05M	6mM	1,065	30
0	0.3M	---	1,367	0	0	0.3M	---	1,180	0
40µg	0.3M	---	957	30	20µg	0.3M	---	940	20
60µg	0.3M	---	858	37	40µg	0.3M	---	871	26

The final 0.2 ml reaction mixtures were as described for Table XXI. 6 mM Spermidine and 0.3 M KCl were not present during the preincubation period but were added at the time of addition of template.

DISCUSSION

Previous to this time, several investigators have reported salt and polyamine-mediated stimulation of DNA-dependent RNA polymerase activity isolated from several microbial sources. Although a considerable number of studies investigating the effect of KCl and other monovalent and divalent salts on many aspects of the in vitro transcription process have been reported, much less has been reported concerning the influence of polyamines on the individual reaction events. The effect of polyamines on certain transcription steps, for example, the termination events, has not been dealt with directly at all. Also, a study adequately comparing the influence of ionic strength and polyamines on in vitro transcription of a well defined DNA template is lacking. This study is an attempt to unify some of the fragmented data dealing with polyamine-mediated stimulation of in vitro transcription by studying and comparing the influence of KCl and polyamines on certain events involved in the transcription of a well defined template isolated from the bacteriophage T4. Although a detailed understanding of the molecular basis of action of polyamines in transcription has not, as yet, been realized, certain definite statements can be made.

The polyamines spermine [H_2N-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2] and spermidine [H_2N-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2] are only moderately active in stimulating in vitro transcription of T4 DNA at low ionic strength (Tables VI and VIII). These polyamines, in the presence of 0.1 M KCl, which alone appears to have little effect, substantially stimulate RNA synthesis (Table I). Spermine and spermidine-mediated stimulation is

also observed at KCl concentrations (0.3 M) near the KCl optimum (Tables V and VII). This polyamine effect at high KCl concentrations was, at first, taken as evidence that polyamines influence transcription in a manner apart from that of KCl. However, a comparison of the effect of KCl and spermidine on individual reaction events required in preventing early cessation of RNA synthesis in no way confirmed this theory.

The apparent inability of either spermidine or spermine to substantially increase *in vitro* transcription of T4 DNA at low ionic strength, can be traced to the problem of establishing the optimal polyamine concentration because of template precipitation, caused by small concentrations of polyamines. A visible precipitate is observed in reaction mixtures containing 0.4 mM spermine or 3.5 mM spermidine. That this precipitate is the DNA template is confirmed by the appearance of a visible precipitate in solutions of DNA upon addition of small amounts of either polyamine, and the disappearance of the precipitate from reaction mixtures containing high concentrations of either polyamine after addition of DNase.

The precipitation of template by polyamines is somewhat dependent upon the nature of the DNA. Precipitation is most readily observed at very low polyamine concentrations with a high molecular weight template, such as T4 DNA, and is observed at higher concentrations of polyamines or, not at all as the template size decreases (Table XIV).

Petersen and Kröger (79) had previously reported protection of calf thymus DNA from precipitation by spermidine by increased ionic strength. Similarly, no precipitate of either T4 DNA or calf thymus DNA by even large concentrations of polyamine has been observed in this present study at intermediate (0.1 M KCl) or high (0.3 M KCl) salt conditions.

The diamines putrescine [H_2N-$(CH_2)_4$-NH_2] and cadaverine [H_2N-$(CH_2)_5$-NH_2] also stimulate in vitro RNA synthesis on T4 DNA (Tables II and IV). Both diamines prevent early plateauing of RNA synthesis and stimulate transcription in the absence of KCl, but at concentration optimums 300 times that of the optimum spermine and 30 times that of the optimum spermidine concentrations. No precipitation of the DNA template is observed even at high (120 - 150 mM) concentrations with these diamines; and, although the presence of putrescine or cadaverine also stimulates RNA synthesis at high KCl concentrations, (Tables III and V) they, unlike the polyamines, show lower optimum stimulatory concentrations in the presence of high concentrations of KCl than at low salt conditions, i.e., 10-30 mM versus 80-100 mM.

Mg^{++}, which also has been reported to stimulate transcription (78), is similar to putrescine and cadaverine with respect to level of stimulation and concentration range at both low and high salt conditions.

It is convenient to arrange these organic and inorganic cations into classes with respect to their influence on in vitro transcription. Class 1 is composed of salts of monovalent cations as represented by KCl. Class 2 is composed of both organic and inorganic divalent cations as represented by putrescine, cadaverine, and Mg^{++}. Class 3 is represented by spermine and spermidine.

The specificity of action of these compounds appears to increase from Class 1 to Class 3 as shown by their relative stimulation to optimum concentration ratios. Also, while Class 2 and Class 3 compounds show stimulation at elevated KCl concentrations, a Class 3 compound, such as spermidine, is not stimulatory at optimum concentrations of a Class 2 compound, such as Mg^{++}, at either high or low KCl concentrations (Tables XII and XIII).

The conclusion that the action of polyamines on transcription can be fully understood by their contribution to the net ionic strength (116) appears to be a hasty one. A large number of experiments have been performed over the past 2 years using a large number of T4 DNA and several RNA polymerase preparations which indicate (Figures 6 and 7) that polyamines stimulate the transcription reaction without appreciably altering the ionic strength of the reaction media. The addition of 2.5 M spermidine to a reaction mixture containing no KCl increases the calculated ionic strength from 0.05 to 0.06 but results in a 3 fold stimulation of transcription, by 240 minutes. Also, the addition of 6 mM spermidine to a reaction mixture containing 0.1 M KCl increases transcription by 10 fold, showing more activity than is observed at an ionic strength of 0.35 (0.3 M KCl), while increasing the calculated ionic strength from 0.15 to 0.18. Furthermore, the addition of 6 mM spermidine to a high salt reaction mixture, raising the calculated ionic strength from 0.35 to 0.38, results in a more than two-fold increase in ^{3}H-UMP incorporation.

Therefore, Class 3 compounds appear to be rather specific in their action on transcription influencing the ionic strength only slightly while Class 2 compounds, although exhibiting lower optimal concentrations than Class 1 compounds, do contribute significantly to the ionic strength. Also, a Class 3 compound in the presence of an intermediate (0.1 M) concentration of a Class 1 compound mimics the effect observed at optimum concentrations of a Class 2 compound. Class 1 compounds appear to be more general than specific affecting other reaction events such as polymerase-DNA association (39, 48).

It has been generally observed in studies dealing with polyamine-nucleic acid interactions, that the effectiveness of polyamines increase in the order putrescine-spermidine-spermine. With respect to this order of effectiveness, parallels can be drawn between the influence of polyamines on in vitro DNA-directed RNA synthesis with other systems where enzyme-nucleic acid interactions are involved such as the replication of DNA by DNA polymerase from rat brain (123), the methylation of tRNA by tRNA methylase (118), and the aminoacylation of tRNA by aminoacyl-tRNA synthetases (119).

Although polyamines are known to bind to nucleic acids (124-129) and influence their thermal stability (120, 125-129), conformational state (120), and solubility (79); the exact nature of their influence on protein-nucleic acid interaction is not clear. It is suggested, however, that the molecular structure of the cation is important since the effectiveness of these organic cations vary with the number of methylene groups and charges, indicating that the influence of different organic and inorganic cations should not be generally categorized as a simple cationic or ionic strength effect.

Kinetic analysis (Figures 8 and 9) of in vitro T4 transcription show little stimulation by KCl or spermidine at early times. Admittedly, these experiments tell very little about the effect of polyamines on the association, initiation, or chain elongation events. They do, however, after a comparison with the effect of polyamines on the overall transcription reaction, indicate that it is the termination events which are most influenced by polyamines. Detailed studies of the effects of polyamines on the association, initiation, and elongation events have not been reported but are clearly called for.

Since the rate of initiation and elongation are not important factors in stimulation of in vitro transcription at later times in the reaction if reinitiation fails to occur, and since the spermidine effect is much more pronounced at later times in the RNA synthetic reaction, it was felt that a study and comparison of the effects of KCl and spermidine on certain termination events should be undertaken.

It has been generally accepted that failure of the RNA polymerase to reinitiate is a result of the failure of nascent RNA to be released from the transcription complex, with the formation of a ternary complex of RNA-enzyme-DNA (23, 37, 89, 94), although some investigators have reported release at low ionic strength (82, 100, 101). Since RNA had been reported to be released at increased ionic strength with KCl (23, 89, 94), it was felt that spermidine would also facilitate release of nascent RNA from the transcription complex and that this might be the mechanism of polyamine-mediated stimulation. Nitrocellulose filter analyses (Figure 10) of the reaction mixtures, with and without spermidine, reveal that nascent RNA is released under both conditions even at low ionic strength (0.05). Thus the failure of nascent RNA to be released, resulting in the formation of an inactive ternary transcription complex, is not responsible for early cessation of RNA synthesis.

Since these results were not consistant with the generally accepted view of "plateau kinetics", sucrose gradient analysis (Figure 11) of reaction mixtures with and without spermidine were also performed. Again, release of RNA was observed at low ionic strength in the absence of spermidine. Furthermore, when the same reaction mixture was analyzed by both methods, the agreement was surprisingly good (Table XVII). There appears to be little doubt that, at least with this system, nascent RNA

is released from the transcription complex and, therefore, spermidine-mediated stimulation is not due to facilitation of nascent RNA release.

It can be suggested, from these studies, that reinitiation occurs in the presence of 2.5 mM spermidine since the amount of total RNA is found to increase while the amount of complex bound RNA remains constant after 30 minutes (Figure 10). Furthermore, a large quanitity of RNA is present in the transcription complex (Figure 11) in reaction mixtures containing 2.5 mM spermidine at times when most of the RNA is released in the absence of spermidine, indicating the presence of an active transcription complex. Whether the polymerase is free, complexed with the DNA or with the RNA once the plateau has been established cannot be determined from these experiments.

It is unclear why release of nascent RNA is observed by some investigators and not by others. However, it is known that Mg^{++} in the gradient buffer over a concentration range of 0 to 4.0 mM, has no effect on the gradient analysis, and spermidine (Table XVII) has no effect on determination of complex bound or total ^{3}H-RNA by nitrocellulose filter analysis.

Spermidine-mediated stimulation is not a result of the synthesis of larger RNA molecules since the size of the T4 DNA transcripts is found not to be affected by ionic strength or the presence of spermidine, as determined by SDS-sucrose gradients (Figure 12) or formaldehyde-sucrose gradients (Figure 13). The molecular weights of the transcripts determined from the formaldehyde-sucrose gradient analysis agree well with those reported by other investigators (89, 100).

Spermidine is also effective in allowing the polymerase to reinitiate on a template other than the one it originally initiated on (Table XIX).

This experiment directly shows that reinitiation occurs in the presence of 2.5 mM spermidine at a calculated ionic strength of 0.06 as well as in the presence of KCl at an ionic strength at which the termination factor, rho, is reported not to be effective (82, 102, 110). It would be of interest to determine whether 2.5 mM spermidine, in a low salt reaction mixture, would provide an environment in which rho-mediated termination and release of RNA can occur as well as reinitiation. That low concentrations of spermidine may provide such an environment, is suggested by the fact that 2.5 mM spermidine is as effective as 0.3 M KCl in allowing reinitiation without appreciably altering the ionic strength.

It is unclear how RNA inhibits the transcription reaction, but, as discussed previously, the failure to release nascent RNA from the transcription complex can be ruled out.

It is very possible that the product RNA reversibly inactivates the polymerase by competing with the DNA for the same binding site or by binding to a site which results in allosteric inactivation of polymerase activity, thus preventing reinitiation. This theory is supported by the observation of polymerase-RNA complex formation (36), of inhibition of RNA synthesis by addition of exogenous RNA to the reaction mixture, (36, 39, 46-48, 51) and displacement of native DNA by tRNA from complexes with the polymerase (48). That there is indeed a competition between RNA and DNA for a binding site on the polymerase is shown by Table XX.

A comparative study of KCl and spermidine in preventing and overcoming inhibition by addition of exogenous RNA (Tables XXI and XXII) shows that both are effective in preventing or overcoming inhibition by 16 S and 23 S rRNA or tRNA. In most cases, 6 mM spermidine appears more

effective than 0.3 M KCl again showing that spermidine is specific in its action and does not act by its contribution to the net ionic strength of the medium. Spermidine also allows reinitiation and, presumably, overcomes product inhibition after it is well established (Figure 14) in a manner comparable with 0.3 M KCl.

One puzzling aspect of this study has been the apparent dependence of the cation mediated-stimulation upon the nature of the template. The presence of 0.3 M KCl in a reaction mixture containing T4 or T7 DNA (Table XV) results in a dramatic stimulation of RNA synthesis, but only modest stimulation is observed with calf thymus DNA, E. coli DNA, or poly [d(A-T)]. Spermidine, (Figure XIV) at low salt conditions, is less effective than 0.3 M KCl with respect to maximum stimulation of transcription of the other three templates. Also, when the template is protected from precipitation by spermidine, by intermediate concentrations (0.1 M) of KCl, T4 transcription is dramatically stimulated while calf thymus DNA is transcribed to a degree similar to that observed with 2.5 mM spermidine alone (Table XVI). In addition, when 6 mM spermidine is present with 0.3 M KCl, transcription of calf thymus DNA is reduced to the level observed when 0.3 M KCl is present alone. It is interesting that the action of these monovalent and polyvalent cations in stimulating transcription of these templates is noticably similar to the influence of sigma factor in stimulating the transcription of these same templates by the core enzyme (33). The significance of this observation is not yet clear.

This study has shown that polyamine-mediated stimulation of in vitro RNA synthesis is not a result of the contribution of the polyamine

to the net ionic strength, facilitation of release of nascent RNA from the transcription complex, or synthesis of larger molecular weight transcription products. Although polyamines are stimulatory at optimal KCl concentrations, there appears to be little difference in the effect of either KCl or spermidine on reinitiation, product RNA size, or restart of the reaction once the plateau phase has been established. Spermidine does appear to be more effective in preventing and overcoming inhibition by exogenous RNA.

Based on these results, the most attractive explanation of polyamine-mediated stimulation of in vitro transcription is that polyamines are specific in preventing or overcoming the reversible inactivation of the RNA polymerase, by product RNA, thereby allowing reinitiation to occur. The inactivation by product RNA is probably due to the competition with DNA for binding sites on the polymerase or an allosteric effect resulting from association of the polymerase with RNA. The apparent ability of polyamines, in the presence of a protecting concentration of monovalent cations, to stimulate RNA synthesis to a greater extent than high concentrations of KCl may be traced to the specific action of polyamines as opposed to the general milieu-altering action of large salt concentration, which may hinder certain reaction events (39, 48).

The inability to make definite predictions of the in vivo action of organic and inorganic cations on RNA synthesis, based on evidence gained by in vitro studies, is apparent because of the difficulty in determining the concentrations of these cations in specific areas of the cell, the influence of active and intracellular transport, and the influence of other cell constituents on the action of these ions. That polyamines do play a role in the in vivo RNA synthesis is supported, however, by many

in vivo studies implicating them, in both a direct and indirect manner, with control of RNA synthesis. The day when evidence obtained from both *in vivo* and *in vitro* studies can be consolidated into a lucid model adequately describing the role of polyamines in RNA synthesis remains in the future.

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